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Identity and Dynamics of the Microbial Community Responsible for Carbon Monoxide Oxidation in Marine Environments

By

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B.S. Biology, emphasis in Marine Biology
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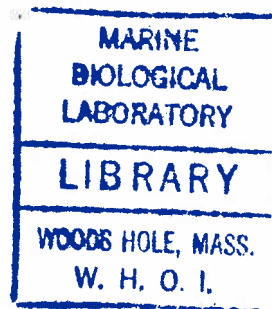
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Submitted to the Department of Biological Oceanography on June 24, 2003
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ABSTRACT

As colored dissolved organic matter in seawater absorbs UV solar radiation, a variety of simple chemical species are produced, including carbon monoxide (CO). The ocean surface water is saturated with respect to CO, and is thus a source of CO to the atmosphere. CO reacts with and removes free-radical compounds, and may itself contribute to the 'greenhouse' gas content of the atmosphere. An important sink for CO in seawater is the biological oxidation of CO to CO₂ by marine microorganisms. The objectives of this study are to identify component members of the microbial community responsible for the oxidation of CO in coastal marine environments through a combination of recent microbiological and molecular approaches, and to estimate their contributions to total *in situ* CO bio-oxidation. We utilize an enrichment method that involves cultivation of bacteria on membrane filters, subsequent incubation with radiolabeled CO, and the use of autoradiography to screen colonies with the desired phenotype. Cell-specific CO-oxidation activity is determined for selected purified strains with a time-series ¹⁴CO-oxidation method. Molecular phylogeny based on 16S-rDNA gene sequence information within the context of the large and growing 16S database determines the phylogenetic relatedness and identity of marine CO-oxidizing bacteria that result from our cultivation program. The CO oxidizing organisms isolated in this study with greatest activity are closely related to the *Roseobacter* and *Paracoccus* genera of the *alpha-proteobacteria*, collectively known as the "marine alpha group". Other microorganisms found to oxidize CO at environmentally relevant rates are members of *beta*- and *gamma-proteobacteria*, and one in the *Cytophaga-Flavobacterium-Bacteroides* group.

A collective CO-oxidation activity was calculated from physiological measurements of purified isolates and abundance estimates of CO-oxidizing marine alpha group organisms. Relative proportions of CO-oxidizing *Roseobacter* and *Paracoccus* cells were resolved microscopically by microautoradiography in combination with DAPI and fluorescent-labeled oligonucleotide probes (Substrate Tracking AutoRadiography – Fluorescent *In Situ* Hybridization (STAR-FISH)). Marine alpha group organisms were a major component of total cell numbers (45.7%) at the time of sampling (March 2003), and CO-oxidizing members of the marine alpha group contributed up to 40.7% of total CO oxidation occurring in coastal waters.

Thesis supervisor: Craig D. Taylor

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“Microbial ecology, the interaction of species with the environment and one another, is process-based, and the phylogenetic positioning of a phylotype can only give hints of the organism's actual function in its environment. Discarding pure culture approaches as too difficult, too slow or unnecessary, or using the term unculturable microbes means throwing the baby out with the bathwater. All those key microbes that make the phylogenetic tree useful appeared to be unculturable until they were cultured, at that point disclosing a wealth of new physiological and biochemical information needed in microbial ecology.”

Holger Jannasch, 1927 - 1998

Chapter I:

General Introduction

Carbon monoxide (CO) is the third most abundant carbon species in the atmosphere, after CO₂ and methane. It is one of the most important reactive gasses in the atmosphere because of its reaction with the hydroxyl radical (OH[•]), which forms a major sink for both atmospheric CO and OH[•] (*Logan et al.*, 1981; *Johnson & Bates*, 1996; *Pos et al.*, 1998; *Zuo et al.*, 1998). Although the dominant sources of atmospheric CO are anthropogenic (*Müller*, 1992; *Conrad & Seiler*, 1982a), it is well documented that sunlight-initiated photodegradation of colored dissolved organic matter (CDOM) is primarily responsible for producing CO in sunlit waters, thus the surface waters of the world's oceans are saturated with CO with respect to the atmosphere and are a source of atmospheric CO (*Swinnerton et al.*, 1970; *Conrad et al.*, 1982).

It is likely that most of the CO produced *in situ* is consumed by microbial activities (*Conrad et al.*, 1982). Great uncertainty exists regarding the source strength of

CO derived from the photodegradation of CO; past studies focused on open ocean water have suggested that the oceanic CO flux may be as high as 200 Tg/y (*Kieber et al.*, 1990; *Mopper et al.*, 1991; *Conrad et al.*, 1982). A more recent study has estimated a global “blue-water” photochemical source of CO, 50 ± 10 Tg carbon from CO per year (CO-C a^{-1}) and its microbial sink, 32 ± 18 Tg CO-C a^{-1} (*Zafiriou et al.*, 2002), suggesting that CO processes are a non-trivial component of the oceanic carbon budget, and most photolytically produced CO is cycled internally by microbial processes. The global CO gas exchange flux has been estimated by *Bates et al.*, (1995) as ~ 5.5 Tg CO-C a^{-1} ; the flux of CO from the ocean into the atmosphere would therefore represent a minor fraction (9% - 14%) of the total CO produced in the ocean (*Conrad & Seiler*, 1980; *Conrad et al.*, 1982). Whether the CO is transferred into the atmosphere or oxidized to CO_2 it represents a direct loss of otherwise refractory DOM carbon in natural waters (*Zuo & Jones*, 1997).

While CO-processes (production and consumption) have been measured on a gross scale in bulk seawater samples for nearly three decades in oligotrophic oceanic waters, there have been no definitive investigations concerning the identity of the microbes responsible for CO bio-oxidation in the marine environment; only circumstantial evidence for CO metabolizing marine organisms based on ancillary ability of certain bacteria to also metabolize CO. This will be the first attempt to both quantitatively and qualitatively describe the organisms responsible for oceanic CO-metabolism, and illuminate the ‘black box’ regarding CO consumption in seawater that has been up to now overlooked in studies of ocean-atmosphere flux of trace gasses and carbon budget models in the oceanic environment.

CO distribution and diel variability

The major source of CO in the surface waters of the ocean is the abiotic photo-oxidation of chromophoric dissolved organic material (CDOM) initiated by UV or near-UV light (Zuo & Jones, 1995, 1997; Pos *et al.*, 1998; Valentine & Zepp, 1993; Mopper *et al.*, 1991). The quantum yields for CO formation were found to decrease in an exponential manner between 280 *nM* to 550 nm. Although the highest quantum yields were observed in the UV-B region (280-320 nm), longer wavelengths of solar radiation, principally in the UV-A region (320-390 nm) and blue spectral region, are most important in inducing CO formation because a greater amount of radiation in these ranges reach the earth's surface (Valentine & Zepp, 1993). A light-induced α -cleavage of carbonyl compounds, a major constituent of natural DOM, may play a primary role in the photoproduction of CO (Pos *et al.*, 1998; Zuo & Jones, 1997). The dependence of CO production on the presence of CDOM, short-wavelength light, and oxygen (Zuo & Jones, 1997; Conrad *et al.*, 1982) and its independence of the presence of plankton or bacteria indicates that CO production is a photochemical rather than photometabolic process. At night photoproduction of CO ceases, thus the nighttime decrease in CO concentration results from two primary mechanisms: sea-to-air exchange and oxidation by microbes (Conrad & Seiler, 1980; Jones, 1991; Johnson & Bates, 1996).

Carbon monoxide concentration in the upper mixed layer in oligotrophic water exhibits a diurnal cycle with increasing concentrations of CO from dawn to a maximum (2-3 *nM*) in middle to late afternoon, followed by a rapid decline in the early evening to a minimum (0.25 - 0.5 *nM*) just before dawn (Johnson & Bates, 1996; Conrad *et al.*, 1982;

Xie et al., 2001). This implies that CO is short-lived and is strongly coupled to the day/night cycle via photoproduction and biology (*Conrad et al.*, 1982; *Jones*, 1991). Daytime vertical profiles of dissolved [CO] show maximum values in the surface water and decreasing [CO] with depth as light in the UV wavelengths is attenuated (*Seiler & Schmidt*, 1974; *Seiler*, 1978; *Jones*, 1991; *Xie et al.*, 2001). The lowest concentration of CO is found below the euphotic zone in depths beyond 100 m where [CO] varies between 1-12 nl l⁻¹ (0.044 – 0.53 nM) (*Conrad et al.*, 1982, lower detection limit of technique was 0.5 nl l⁻¹ or 0.022 nM), and below detection limits at 200m (<0.03 nM) (*Xie et al.*, 2002b). The diel variation of dissolved CO occurs in the entire euphotic zone, with decreasing amplitude at greater depths reflecting decreasing light intensity. A phase lag of the diel variation within the euphotic zone – indicative of vertical CO transport – is lacking. In contrast with conditions within the euphotic zone, CO concentration varies little with time below the mixed layer. This indicates that the net exchange of CO between the euphotic zone and the underlying deep ocean water is very small and cannot account for the observed diurnal variation within the euphotic zone (*Zuo & Jones*, 1997; *Conrad et al.*, 1982; *Jones*, 1991; *Zuo et al.*, 1998).

Microbial groups implicated in CO oxidation

Several biogeochemical studies have estimated the diel, annual, and global rates of microbial CO oxidation as an oceanic CO sink (*Conrad & Seiler*, 1980, 1982b; *Conrad et al.* 1982; *Johnson & Bates*, 1996; *Jones*, 1991; *Xie et al.*, 2002; *Zafiriou et al.*, 2002; and citations within) but few have attempted to investigate beyond the “black-box”

level of the phenomena occurring in marine environments (Jones & Amador, 1993; Jones & Morita, 1983b, 1984). There are no definitive investigations concerning the identity of the microbes responsible for CO bio-oxidation in the open-water marine environment; only circumstantial evidence for CO metabolizing marine organisms based on ancillary ability of certain bacteria to also metabolize CO. Two strains of ammonium-oxidizing microbes (*Nitrosococcus oceanus* and *Nitrosomonas europaea*) isolated from seawater and strains of methanotrophic (*Pseudomonas methanica*) bacteria isolated from brackish sediments have been shown to possess the ability to oxidize trace amounts of CO to CO₂ in pure-culture studies, though aerobic methane and ammonium-oxidizing bacteria catalyze the oxidation of CO only when metabolizing other substrates and at the expense of the cell's reducing power (Ferenci et al., 1975; Jones & Morita, 1983a, b).

A phylogenetically diverse group of microorganisms isolated from terrestrial and freshwater soils has been identified as having the ability to survive high concentrations of CO and a metabolism that allows growth with CO as their sole source of carbon and energy (Meyer and Schlegel, 1983; Meyer et al., 1986; Nozhevnikova & Yurganov, 1978; Zavarzin & Nozhevnikova, 1977). While CO-utilizing or “carboxydophilic” bacteria have been successfully isolated from marine hydrothermal environments (Svetlichny et al., 1991a, 1991b; Sokolova et al., 2001; Gerhardt et al., 1991), these organisms, without exception, are strictly anaerobic and extremely thermophilic. These are therefore unlikely contributors to the observed CO consumption in marine surface waters.

Microbial Metabolism of CO

Bacteria apparently contribute to CO consumption rather than production. Size fractionation experiments with ocean and lake water have shown that the CO oxidizing microorganisms are in a size range of 0.2-3.0 μm (Conrad *et al.*, 1982; Conrad & Seiler, 1982b; Olson, 1981) that excludes most of the eucaryotic phytoplankton population (Williams, 1981). There are two opposing views concerning the organisms responsible for the oxidation of CO in natural samples. Conrad and Seiler (1982b) suggest that the utilization of CO (in eutrophic lake water) is due to specific organisms that gain distinct advantage from the oxidation of CO. Evidence for this comes from low K_m values (7 - 9 nM) and use of CO at trace levels (~ 0.04 nM) by the organisms reported in their studies. Bartholomew & Alexander (1979) suggested that CO oxidation (in soil suspensions) is due to microorganisms that utilize CO gratuitously by co-oxidation or co-metabolism, that is, the transformation of a non-growth substrate in the obligate presence of a growth substrate (Dalton & Stirling, 1982). This view is supported by their findings that essentially no radioactivity from ^{14}C -CO appeared in the organic fraction under their experimental growth conditions and that ^{14}C -CO₂ fixation was not enhanced in the presence of CO. These concepts are not mutually exclusive; groups of organisms that satisfy one or the other of these criteria may contribute jointly to total CO oxidation observed in natural waters.

Several workers have suggested that nitrifying bacteria contribute to CO oxidation in the oceans (Jones & Amador, 1993; Conrad & Seiler, 1980). A number of marine ammonium- and methane-oxidizing proteobacteria are identified as having a K_m for CO

in the nanomolar range and the ability to oxidize CO at *nM* concentrations in pure or enriched cultures. Methanotrophic bacteria are able to oxidize CO at trace levels and this oxidation is linked to the lack of specificity of the enzyme methane monooxygenase (MMO) (Ferenci *et al.*, 1975). Due to the close similarity of the ammonium monooxygenase (AMO) and MMO enzymes, ammonium-oxidizing bacteria are also capable of CO oxidation at low CO concentrations in the environment. Jones and Morita (1983a) demonstrated that methane is a utilizable substrate for ammonium oxidizing bacteria, further demonstrating the similarity. CO is known to be a competitive substrate for AMO, and inhibits NH_4^+ oxidation in the marine environment at concentrations ranging from 2 *nM* to 11.4 μM (Ferenci, 1974; Jones & Morita, 1983b; Vanzella *et al.*, 1989). The ammonia oxidizers *Nitrosomonas europaea* and *Nitrosococcus oceanus* are able to oxidize CO to CO_2 in the presence and absence of ammonium, but attempts to grow ammonium oxidizers on CO as the sole source of carbon and energy failed. None of the organisms tested incorporated any of the carbon from CO into cellular components in the absence of ammonium, but all were able to oxidize CO at trace concentrations (<0.5 *nM*). Although ammonia oxidizers can oxidize CO in the absence of ammonium, the oxidation can be considered to be a co-metabolism or “co-oxidation” (Dalton & Stirling, 1982) since carbon from CO is not incorporated into cell material (Jones & Morita, 1983b, 1984). CO oxidation in these organisms is a gratuitous process that actually drains the cell’s supply of reducing power and does not support the growth of either methane- or ammonium-oxidizing organisms.

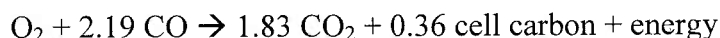
Terrestrial aerobic microorganisms able to utilize carbon from CO include the chemolithoautotrophic carboxydobacteria (Zavarzin & Nozhevnikova, 1977; Meyer, 1985; Meyer & Schlegel, 1983), and other species of fungi and algae (Bartholomew & Alexander, 1979; Inman *et al.*, 1971). “Carboxydotrophic bacteria” or “carboxydobacteria” is a convenient operational term for the designation of organisms capable of oxidizing CO and growing chemolithotrophically. The group of carboxydotrophic bacteria defined by physiological characteristics is taxonomically diverse and is comprised of species of many different genera, e.g. *Pseudomonas*, *Alcaligenes* (*Hydrogenomonas*), *Bacillus*, *Arthrobacter*, *Azomonas*, and *Azotobacter*, *Achromobacter*, *Comamonas*, *Seliberia* (Nozhevnikova & Zavarzin, 1974). From the diversity of carboxydobacteria observed it follows that it is unreasonable to revive a separate genus for lithotrophs oxidizing CO. The genus *Hydrogenomonas* was rejected by Davis *et al.* (1970) and the habit of grouping organisms according to similarity of physiological characteristics has disappeared (Nozhevnikova & Zavarzin, 1974).

Enrichment and isolation of carboxydotrophic bacteria from natural habitats are traditionally done in liquid batch cultures incubated aerobically in desiccators supplied with CO₂ and CO as carbon and energy sources. Such conditions are highly specific for the development of carboxydotrophs because of the inability of the contaminating microflora to utilize CO, and inhibition of respiratory oxidases by irreversible binding of CO in the vast majority of organisms results in inhibition of their growth (Meyer & Schlegel, 1983). Carboxydotrophic bacteria have been isolated from various freshwater soil and water sources and are able to utilize CO as an energy and carbon source, and are

also able to grow like hydrogen bacteria on H_2 , O_2 , and CO_2 (Zavarzin & Nozhevnikova, 1977; Wood, 1991, Meyer & Schlegel, 1983). Many possible habitats for carboxydobacteria are identical to those of hydrogen bacteria (Nozhevnikova & Yurganov, 1978), including geothermal environments (Lilley *et al.*, 1982), composting materials (Conrad, 1988) and submerged soils or soils with stagnant water (Inman *et al.*, 1971; Rich *et al.*, 1998). When pure cultures of other hydrogen bacteria were studied for their ability to utilize CO, the results were negative; hydrogen bacteria were generally very sensitive to CO (Zavarzin & Nozhevnikova, 1977).

The ability of the known carboxydobacteria to tolerate high [CO] stems from a branched respiratory chain, the autotrophic branch of which contains a CO insensitive *o*-type cytochrome (Meyer & Schlegel, 1983, Kim & Hegeman, 1983) or cytochrome b_{653} (Meyer *et al.*, 1986) functioning as terminal oxidase. CO:acceptor oxidoreductase [carbon monoxide oxidase (COX) (Meyer & Schlegel, 1983) or carbon monoxide dehydrogenase (CO-DH) (Meyer *et al.*, 1986, Kim & Hegeman, 1983)], the key enzyme in energy metabolism of these bacteria, is a selenium-containing molybdo-iron-sulfur-flavin hydroxylase. COX or CO-DH serves three functions: (a) to feed electrons into the respiratory chain for electron transport phosphorylation, (b) to provide the carbon source as CO_2 , and (c) to provide ATP and, by inverse electron transfer, NADH for CO_2 assimilation via the reductive pentosephosphate cycle. Carboxydotrophs oxidize CO for generation of energy, $5 CO + 2.5 O_2 \rightarrow 5 CO_2$, and of reducing equivalents, $2 CO + 2 H_2O \rightarrow 2 CO_2 + 2 \times 2[H]$. CO_2 derives from the oxidation of CO and is assimilated via the ribulosebiphosphate cycle: $CO_2 + 2 \times 2[H] \rightarrow (CH_2O) + H_2O$. The sum of these

equations, $7 \text{ CO} + 2.5 \text{ O}_2 + \text{H}_2\text{O} \rightarrow 6 \text{ CO}_2 + (\text{CH}_2\text{O})$, indicates one-seventh of the CO-born carbon being assimilated and is in accordance with the actual stoichiometry of uptake and fixation of CO by growing cells of *Pseudomonas carboxydovorans* and other carboxydotrophs:



Under these conditions, the electrons derived from the oxidation of CO are channeled into the CO insensitive branch of the respiratory chain. The amount of CO used for energy generation may be much higher under oligotrophic or nutrient-poor growth conditions; therefore, the amount of CO-born CO₂ assimilated by different strains ranges from 2 – 16% (Meyer & Schlegel, 1983, for review).

There seems to be considerable variation in the efficiency with which CO-derived CO₂ is assimilated according to growth conditions and among different strains of carboxydobacteria. This range indicates poor coupling in some strains between the CO oxidation and CO₂ fixation processes and may reflect some variation among the electron transport systems of carboxydobacteria. It seems clear that CO-dependent growth is not very efficient in most cases, although the highest assimilation ratios agree well with the theoretically expected values (Meyer & Schlegel, 1979, 1983; Hegeman, 1984)

In isotopic experiments with ¹⁴C-CO, ¹⁴C-HCO₃⁻, and ¹⁴C-CO₂, various species of carboxydobacteria incorporated labeled carbon into acid-stable products within the cells. However, the kinetics of accumulation of the label by the cells differed. The cells incubated in an atmosphere of unlabelled CO and ¹⁴C-HCO₃⁻ began to assimilate carbon dioxide at once and at a constant rate; moreover, the presence of CO as an energy

substrate was necessary. The labeled carbon from ^{14}C -CO, in the absence of exogenous bicarbonate, was incorporated in the acid-stable products of the cells after a lag period, with the formation of acid-unstable intermediate(s). The addition of unlabeled carbon dioxide reduced the rate of incorporation of the label from ^{14}C -CO. The same was observed when ^{14}C -CO₂ formed from labeled CO was absorbed by NaOH or KOH (Nozhevnikova & Yurganov, 1978; Zavarzin & Nozhevnikova, 1977).

The key enzymes of the Calvin cycle have been found to be active in different strains of carboxydotrophic bacteria grown on CO. That carboxydotrophic bacteria growing on CO actually use the Calvin cycle for CO₂ fixation *in vivo* and that CO is assimilated after its conversion to CO₂ is evident from the following findings: (a) The activities of rubulosebisphosphate carboxylase and phosphoribulosekinase are in the same order of magnitude during growth with CO or H₂ + CO₂ (Kiessling & Meyer, 1982) and are, therefore, high enough to allow the observed growth rates with CO. (b) Very thin suspensions of carboxydotrophic bacteria do not grow with CO unless CO₂ is present (Meyer & Schlegel, 1978). (c) Growth with CO and the rate of ^{14}C -CO assimilation are decreased by the presence of KOH (Nozhevnikova & Yurganov, 1978; Zavarzin & Nozhevnikova, 1977). (d) Labeled CO was assimilated after a lag period only, whereas ^{14}C -CO₂ was incorporated instantaneously (Nozhevnikova & Yurganov, 1978; Zavarzin & Nozhevnikova, 1977). (e) Dilution of ^{14}C -CO by CO₂ resulted in a significant decrease of label incorporated into cell material (Nozhevnikova & Yurganov, 1978; Zavarzin & Nozhevnikova, 1977). Furthermore, the analysis of early fixation products from ^{14}C -CO and ^{14}C -CO₂ of *Pseudomonas carboxydohydrogena*, *Pseudomonas gazotropha*, and

Alcaligenes carboxydus were identical; after 3 sec of incubation, about 40% of the radioactivity was found in phosphoglycerate, the rest was found in phosphorylated sugars (Zavarzin & Nozhevnikova, 1977).

The carboxydobacterium *Pseudomonas carboxydoflava* is remarkable because it has a constitutive CODH and oxidizes CO during heterotrophic growth, i.e. mixotrophically (Kiessling & Meyer, 1982). Under these conditions, which approximate natural conditions better than purely autotrophic growth at the expense of CO, there is little or no assimilation of CO-derived CO₂, apparently owing to repression of the reductive pentose cycle. However, the presence of CO increases the cell yield, demonstrating that there is at least an energetic benefit to the oxidation of CO.

The question of which organisms are performing CO bio-oxidation observed in coastal or oceanic surface waters has not been satisfactorily resolved. As the marine microflora are thought to be actively oxidizing CO, the main problem is to find out if there exists a specialized group of microorganisms that is capable of utilizing CO at environmental concentrations, or if CO at environmental concentrations is oxidized by all or most members of the microbial assemblage present in the environment. This work provides the first definitive identification of the CO metabolizing microorganisms that exist in a coastal marine environment. Through a combination of recent microbiological and molecular techniques, some component members of the CO metabolizing community are identified and their phylogenetic relationships determined in the context of the large and growing 16S-rRNA database.

OBJECTIVES

The following investigations undertook the isolation, identification and phylogeny of important members of the microbial community responsible for oxidation of CO in the coastal marine environment, and determined the relative contributions of these organisms to total CO oxidation observed in natural waters in a multi-phase research program (figure 1.1).

Objective 1: Isolation and Culture of marine CO oxidizing microorganisms.

Our isolation protocol allowed the simultaneous recovery of CO metabolizing organisms with different growth rates and nutritional requirements. While there is ample evidence for the existence of carboxydophilic bacteria in terrestrial soils and wetlands, there have been no documented attempts to obtain or enumerate CO-utilizing microbes in marine (non-hydrothermal) environments. Growth conditions were provided for the cultivation of marine organisms capable of CO metabolism under elevated (~40 nM) dissolved CO concentrations relative to the natural environment. Microbial samples from the environment were incubated on separate mineral media containing either NO_3^- or NH_4^+ as alternate nitrogen sources, permitting the discrimination of strains that incorporate carbon from CO from those that oxidize CO by co-oxidation. Autoradiography permitted rapid screening of CO metabolizing strains amidst a potential background of other colonies. Purified strains were tested individually for their CO-oxidizing phenotype and their activity rates determined.

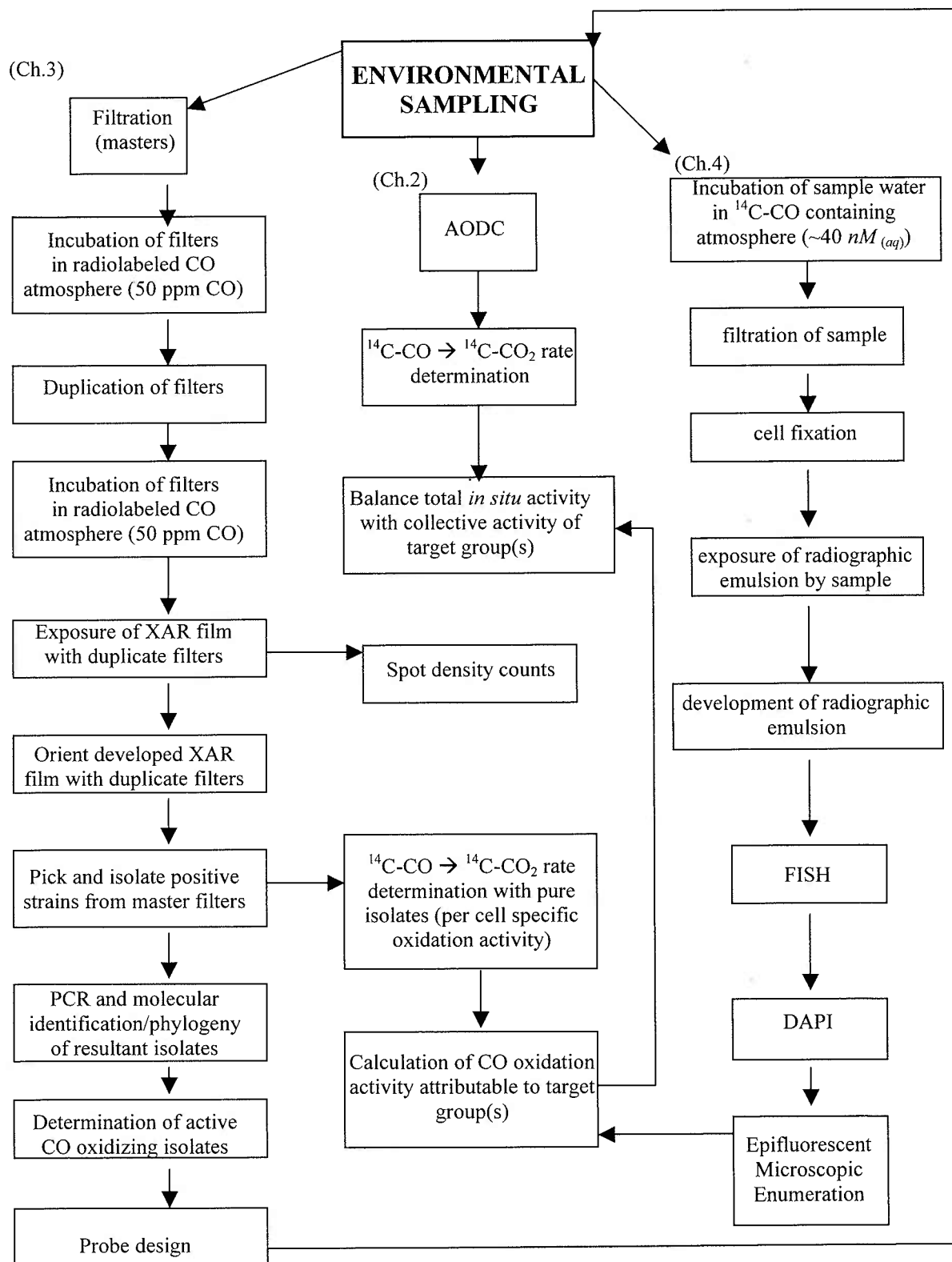
Objective 2: Identification and phylogeny of CO oxidizing isolates based on small subunit ribosomal gene sequence.

With CO metabolizing strains isolated during the cultivation phase, we determined their 16S-rDNA sequence information by PCR amplification using general eubacterial primers. We compared the sequence information obtained from selected isolates within the context of published 16S rRNA gene sequences to determine their phylogenetic relationships.

Objective 3: Enumerate CO oxidizing organisms in natural samples

We estimated the proportion of cells in the natural marine environment that metabolize carbon from CO, and furthermore determined the proportion of CO metabolizing cells on a group-(genus)-specific level, by combining microautoradiography with group-specific fluorescent *in situ* hybridization with oligonucleotide probes developed based on the genetic identity of resultant strains. The substrate-tracking microautoradiography – fluorescent *in situ* hybridization (STAR-FISH) assay is a triple-labeling technique that allowed simultaneous microscopic visualization of i) generally labeled cells (DAPI), ii) cells that have incorporated labeled carbon from CO and have exposed radiographic emulsion, and iii) cells which have hybridized with group-specific fluorescent-labeled oligonucleotide probes.

Figure 1.1: Flow scheme of overall thesis program, showing the methods that allow isolation, specific activity measurement, and differential quantification of phenotype in natural samples. Major research areas are separated into Chapters.



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CHAPTER II:

Biological CO Oxidation Rates in the Sargasso Sea and in Vineyard Sound, MA.

ABSTRACT

Needed in microbial ecology are improved methods for measurement of microbial metabolic activity in natural waters under conditions approaching those *in situ*. Here we report kinetic parameters for CO oxidation for microbial assemblages in open ocean (Sargasso Sea) and coastal marine environments (Vineyard Sound, MA), and directly compare techniques to measure CO loss rates and CO oxidation rates in natural samples. Gross CO oxidation rates in the surface waters of the Sargasso Sea in the summer 1999 cruise (dark incubation rate coefficient) ranged between 0.019 to 0.028 hr⁻¹. These rates were significantly higher than the *in situ* rates for that season (0.0017 – 0.0025 hr⁻¹), and also both the dark incubation and *in situ* rates for the spring 2002 season which ranged between 0.0083 and 0.017 hr⁻¹ and 0.0041 - 0.0062 hr⁻¹, respectively. We find that oxidation of CO in the Sargasso Sea obeys first order kinetics up to 3.5 – 4.5 nM [CO] in closed incubations; incubations containing higher [CO] obey zero-order or saturation kinetics. Furthermore, CO oxidation rates obtained in dark incubations are not applicable to the entire photic period; the rates of CO oxidation determined *in situ* in daytime are depressed by up to an order of magnitude compared with dark incubations. This may result in overestimating the diel rate by up to 85% if it is based on solely on dark incubations, as has been the common practice. The specific CO oxidation activity of the Sargasso Sea assemblage averaged $1.3 \times 10^{-11} \pm 2.9 \times 10^{-12}$ nmol CO oxidized cell⁻¹ hr⁻¹ in the summer and $2.8 \times 10^{-11} \pm 3.2 \times 10^{-12}$ nmol CO oxidized cell⁻¹ hr⁻¹ in the spring.

INTRODUCTION:

The diurnal scale of variability in aquatic CO in the surface waters of the world's oceans is imposed primarily by the source, i.e. production by solar photolysis of chromophoric dissolved organic matter (CDOM). Large diurnal amplitudes require fast acting sinks that are provided by microbial oxidation (Conrad & Seiler, 1980; Conrad & Seiler, 1982b; Conrad *et al.*, 1982; Jones & Morita, 1984; Jones & Amador, 1993), and gas exchange (Conrad *et al.*, 1982; Conrad & Seiler, 1982b; Zuo & Jones, 1995; and many others). CO concentration increases from dawn to a maximum (2-3 nM) in middle to late afternoon, followed by a rapid decline in the early evening to a minimum (0.25 - 0.5 nM) just before dawn (Johnson & Bates, 1996; Conrad *et al.*, 1982; Xie *et al.*, 2001)

(Figure 2.14). This implies that CO is short-lived and is strongly coupled to the day/night cycle via photoproduction and biology (Conrad *et al.*, 1982; Jones, 1991).

Microbial CO loss rates have typically been measured in dark incubations spanning 12 - 20 hours (Jones, 1991; Jones & Amador, 1993; Jones *et al.*, 1984; Johnson & Bates, 1996). Dissolved [CO] within each subsample is measured by a headspace method using a Trace Analytical RGA-3 detector (Xie *et al.*, 2002a). Since CO loss kinetics are first-order (Jones & Morita, 1984; Jones & Amador, 1993; Johnson & Bates, 1996), exponential decays are fitted to the time series data to obtain a CO loss rate constant, k_{co} (Zafiriou *et al.*, 2002). This method yields net CO loss rate constants at [CO] slightly below the sample's initial concentration. The more commonly used ^{14}C -CO method (Jones, 1991; Jones & Amador, 1993; Griffiths *et al.*, 1982) yields potential CO oxidation rates when dissolved [CO] within the incubation is two- to ten-fold above oceanic values, as additional CO is introduced as a tracer substrate. If these incubations have elevated [CO] within the region of first-order kinetics, they are hence applicable to calculating k_{co} values.

In this study, we compare the results of two different methods of determining microbial CO oxidation rate. We report environmental CO oxidation rates by microorganisms in an oceanic oligotrophic environment (Bermuda Atlantic Time Series site), and in a coastal environment in southeastern Massachusetts. We utilize a new ^{14}C -CO tracer technique that allows the determination of CO oxidation rates in the presence of CO photoproduction, and compare traditional dark-incubation CO oxidation rates with rates determined *in situ* in daytime incubations.

METHODS/MATERIALS

Fieldwork was conducted during cruises EN327 and EN335 in waters near the BATS site in August, 1999 and March 2000, respectively. The ship was intended to remain approximately in the same water masses by following a free-drifting drogue and optical buoy array for *in situ* incubations (described below). EN327 was occupied from Year Day (YD) 218 at 31.37°N, 64.00°W to YD 229 at 31.50°N, 64.03°W, and EN335 started on YD 77 at 32.01°N, 64.02°W and ended on YD 88 at 32.03°N, 64.01°W. Front passage or deviation from the Lagrangian mode was monitored by continually profiling sea surface temperature (SST), salinity, and chlorophyll fluorescence and by recording the current vector vs. depth and time via an ADCP. The timing of the two cruises (winter vs. summer) was intended to maximize expected differences in CDOM quantity and the range of key variables, such as wind speed, biology, mixed layer and euphotic zone depths (Xie et al., 2001).

Coastal sampling; Vineyard Sound, MA

The primary coastal sampling site for this study was at the WHOI Shore Lab pier located 1 km east of Nobska Light, Woods Hole, on a southeast-facing beach on Vineyard Sound, Cape Cod, in Massachusetts (Figure 2.1). This location was chosen for its proximity to the laboratory, ease of sampling, and distance from coastal pond- and freshwater discharge. Syringe or bottle samples were collected 0.5 m below the surface to avoid atmospheric CO contamination, in water depths of approximately 1 m.

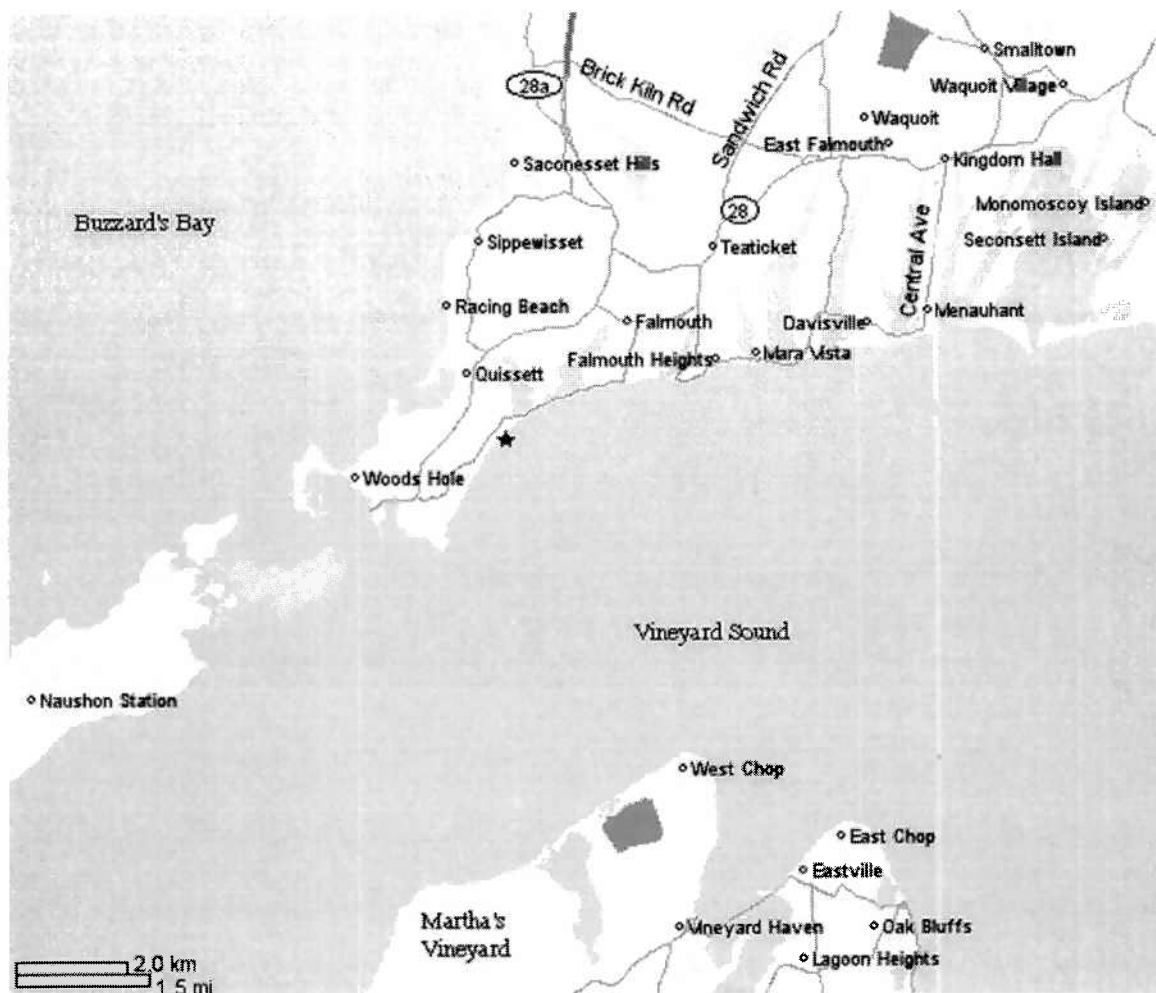


Figure 2.1: Coastal sampling site (Chemotaxis Dock) in Vineyard Sound, MA denoted by star (★)

Generation of ^{14}C -labeled CO.

^{14}C -labeled CO was generated aboard ship by dehydration of 100 μCi of ^{14}C -labeled formate (56 mCi/mmol, 7:3 ethanol:water, ICN Biomedical) with 1.0 ml 36N anhydrous sulfuric acid in a sealed 120 ml serum vial. The formate/acid mixture was kept at room temperature with frequent agitation, and the reaction proceeded to completion after 24 hours. The mixture in the serum vial was made alkaline with the addition of 5.0 ml 10N NaOH to sequester ^{14}C -labelled CO_2 that may have been produced during the acid-dehydration procedure. ^{14}C -CO activity in the stock bottle was monitored periodically (after every 15 - 20 ml removed) by injecting a 1 ml aliquot with a gas-tight syringe into a $>400^\circ\text{C}$ heated copper column (.6 cm ID x 20 cm) containing copper oxide chaff as a catalyst to oxidize CO to CO_2 . Zero-grade compressed air was used as the carrier gas, with flow-rate of approximately 60 ml min^{-1} . The ^{14}C - CO_2 product was captured quantitatively by bubbling through a paired series of scintillation vials containing a mixture of 10.0 ml scintillation cocktail (Scintiverse II) and 0.2 ml hyamine hydroxide (ICN Biomedical, Inc.) downstream from the catalyst. Vial activities were measured via liquid scintillation spectroscopy in the ship's or shore-based laboratory. Most labeled CO_2 product ($>99\%$) was captured in the first vial of the series, and the activity of the two vials was summed for total activity. Activities in the second vial were often indistinguishable from background levels (30-40 DPM) of activity. The linear decrease in activity in the fixed-volume stock vessel with successive gas aliquot removal was applied to calculate the activity (DPM) injected into each sample incubation vessel, and therefore the ^{14}C -CO specific activity within the incubation ($\mu\text{Ci}/\mu\text{mol}$).

¹⁴C-CO oxidation assay

Samples were collected in standard 10-L Niskin bottles fitted with silicon O-rings and Teflon[®] coated springs, or in custom-built titanium bottles (Doherty *et al.*, 2003) mounted on a CTD rosette. Water was drawn from the bottles without contact with the atmosphere in 100 ml ground-glass syringes after three complete flushes with sample water. Sub-samples (25.0 ml) were dispensed into 130 ml quartz vials for the *in situ* incubations and into 75 ml borosilicate glass serum vials for dark incubations. The bottles were sealed with rubber septa. Aliquots (1.0 ml) of ¹⁴C-CO stock were withdrawn with a gas-tight syringe and injected into replicate pairs of sample-incubation vials. The quartz bottles for *in situ* incubations provided a relatively large (>100 ml) headspace that provided a large reservoir of CO that was in equilibrium with CO that was dissolved in the aqueous phase. A potential doubling of aqueous [CO] due to photoproduction, when equilibrated into the gas reservoir, resulted in <5% change in ¹⁴C-CO specific activity and hence, measured rates. Ambient CO concentration in the room air at the time of sealing was determined with a reduced gas analyzer (Trace Analytical). Samples were incubated in the dark within 2°C of the *in situ* temperature. The incubation was stopped by injecting 1.0 ml of 10 N NaOH to raise the pH of the sample to >9, and shaken to sequester any CO₂ present in solution or the headspace. Zero-time bottles were fixed by injecting NaOH within 5 minutes of injecting with ¹⁴C-CO, and for dark-incubated samples, time-point samples (duplicates) were fixed with NaOH at three-hour intervals. The NaOH-stabilized samples were then stored until ¹⁴C-activity could be assessed at a shore-based laboratory. On shore, sample incubation vials were opened to allow

unreacted $^{14}\text{C-CO}$ to exchange overnight. The vials were resealed with a CO_2 -trapping wick (fluted filter paper treated with 0.2 ml hyamine hydroxide) suspended in the headspace above the liquid. The samples were acidified by injection of 1.0 ml 3.6 N H_2SO_4 to lower the pH below 2, and left overnight on a shaker-table at 100 rpm. The CO_2 -traps containing the product of microbial oxidation of $^{14}\text{C-CO}$ were placed in a scintillation vial containing 10.0 ml Scintiverse II and measured for activity by liquid scintillation spectroscopy (figure 2.2). DPM for each vial was corrected for the mean t-zero background ($n=2$), and nanomoles of CO_2 produced in each vial was calculated by dividing by the specific activity of the $^{14}\text{C-CO}$. This was converted to a nanomolar value (in 25 ml sample), and the rate of oxidation over the incubation period was determined (Griffiths *et al.*, 1982; Jones *et al.*, 1984). First order rate coefficients were calculated by normalizing the time-series rate of CO oxidation to CO_2 (nM/hr) by the total dissolved $[\text{CO}]$ (nM) within each incubation vessel to yield the rate coefficient (k_{co}) expressed in reciprocal hours (hr^{-1}). Determining the total dissolved $[\text{CO}]$ required the Bunsen solubility coefficient (β) for CO (Weisenburg & Guinasso, 1979), which is a function of temperature and salinity during the incubation*.

$$* \ln \beta = A_1 + A_2(100 / T) + A_3 \ln (T/100) + S\%[B_1 + B_2(T/100) + B_3(T/100)^2],$$

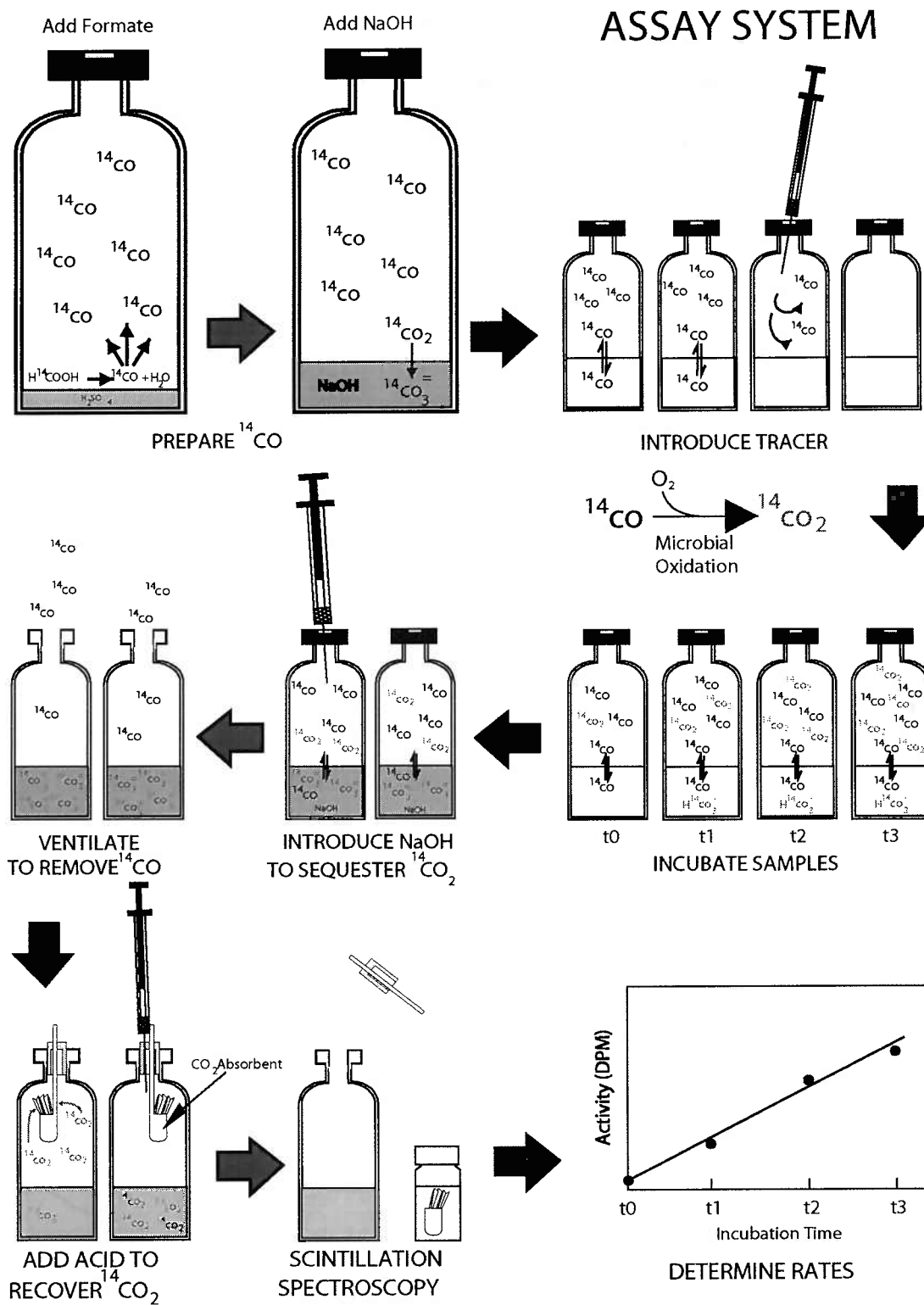
where A_i and B_i are constants, T is the temperature in degrees Kelvin, and S‰ is the salinity in parts per thousand.

$$A_1 = -47.6148, A_2 = 69.5068, A_3 = 18.7397$$

$$B_1 = 0.045\ 657, B_2 = -0.040\ 721$$

(Weisenburg & Guinasso, 1979)

Figure 2.2: Scheme of CO oxidation rate assay. ^{14}C -CO is produced by acid dehydration of ^{14}C -formate, and injected into the headspace of sealed sample vessels to incubate. The product of CO oxidation is quantitatively captured and counted for activity by liquid scintillation spectroscopy (figure courtesy C. Taylor).



Total CO within each incubation vessel included the *in situ* dissolved [CO], the ambient CO of the lab atmosphere when the vial was sealed, and the labeled CO injected prior to incubation. Where it was determined that CO oxidation activity was at maximum velocity because of the effects of CO saturation, all rates from incubations containing higher total [CO] concentrations than at V_{\max} were normalized by [CO] at V_{\max} (4.5 nM CO and 3.5 nM CO for EN327 and EN335, respectively). This correction zero order kinetics is further explained in the Discussion.

In Situ CO Measurements.

[CO] in surface water supplied by a retractable boom (Donoghue *et al.*, 2001), Niskin bottle, or ship's pumping system was continuously monitored by an automated air-segmented continuous-flow-equilibration system developed by Xie *et al.* [2002a]. In brief, precisely regulated flows of CO-free air and of seawater or an aqueous CO standard were injected into two temperature-buffered glass coils (6.1 m in length and 4 mm in I.D.) (figure 2.3-A) in a manner forming discrete gas/liquid segments (figure 2.3-B). The volume ratio of gas to liquid in the coil was ~1:11, and the coil residence time was ~7 min. The partially CO-equilibrated gas was automatically injected into a Trace Analytical Reduction Analyzer (RGA3) for CO quantification. The analytical blank and precision were 0.02 nM and $\pm 2.5\%$ (n = 10, at ~1 nM level), respectively and the accuracy of this method was better than 5%. The system was able to process 4-6 samples per hour. Operation of the system and data acquisition were automated using a LabView[®]-coded program (Wei Wang, WHOI Dept. Chemistry; Xie *et al.*,

2002a). Discrete samples from CTD casts, *in-situ*, and laboratory incubations were analyzed using a head-space method [Xie *et al.*, 2002a]. Briefly, 5-mL of CO-free air was dosed into the water-filled syringes, forming a 1:18 gas/water ratio. Equilibration between gas and water was achieved by vigorous mechanical shaking for 4 min. and the equilibrated head-space gas was injected into a modified RGA3 for CO quantification. The system was standardized using a moisturized (100% relative humidity) 1.23-ppm gaseous CO standard. The analytical precision and accuracy were ± 0.02 nM ($\pm 2\%$) and better than $\pm 10\%$, respectively. The system responded linearly up to ~ 12 nM, and could process ~ 4 -6 samples per hour. The RGA3 had a 2.0 ml loop which was flushed by direct injection of 5.0 ml gas samples.

In situ CO oxidation rate determination: Optical Buoy.

For the *in situ* daytime incubations, pairs of quartz vials were prepared as previously described and incubated at discrete depths using an optical buoy developed for this purpose (figure 2.4). The buoy was a cross-braced tetrahedron of 1" Al pipe 3m on a side whose base corners were floated by small, paired Norwegian floats. The apex bears a radio transmitter beacon, strobe light, and pennant. A central sample-line attachment point was ~ 0.7 m above the water surface and 2m from the floats, minimizing shadowing. Samples in paired 130ml quartz flasks were attached neck-down in light, strong stainless steel cages at adjustable, known depths to a weighted wire rope. Light-shadowing was estimated to average $< 5\%$. Buoy deployment occurred at dawn and recovery at dusk.

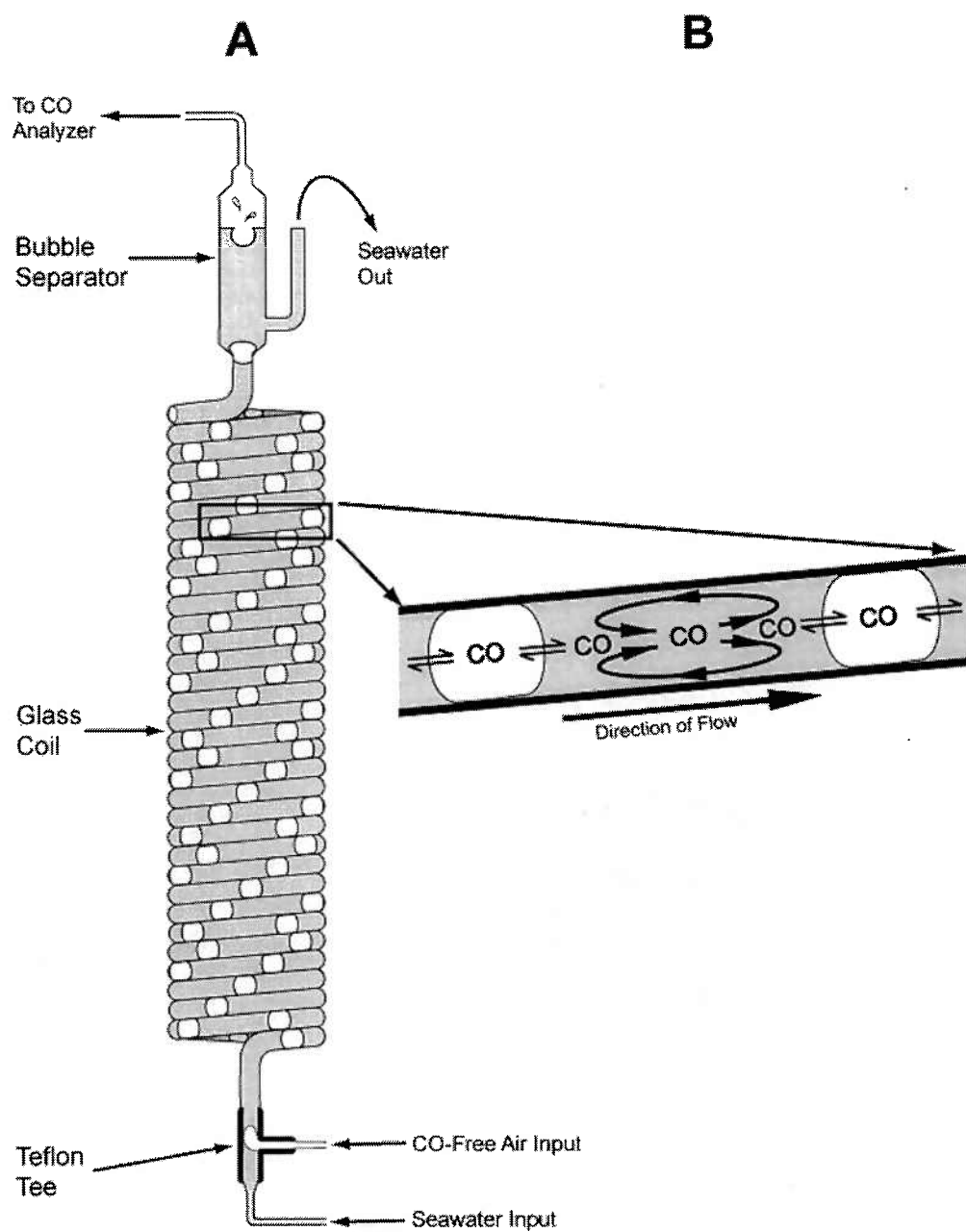


Figure 2.3: Automated air-segmented continuous-flow-equilibration system to monitor [CO] in oceanic surface water (*from Xie et al., 2002a*). The system was able to process 4-6 samples per hour.

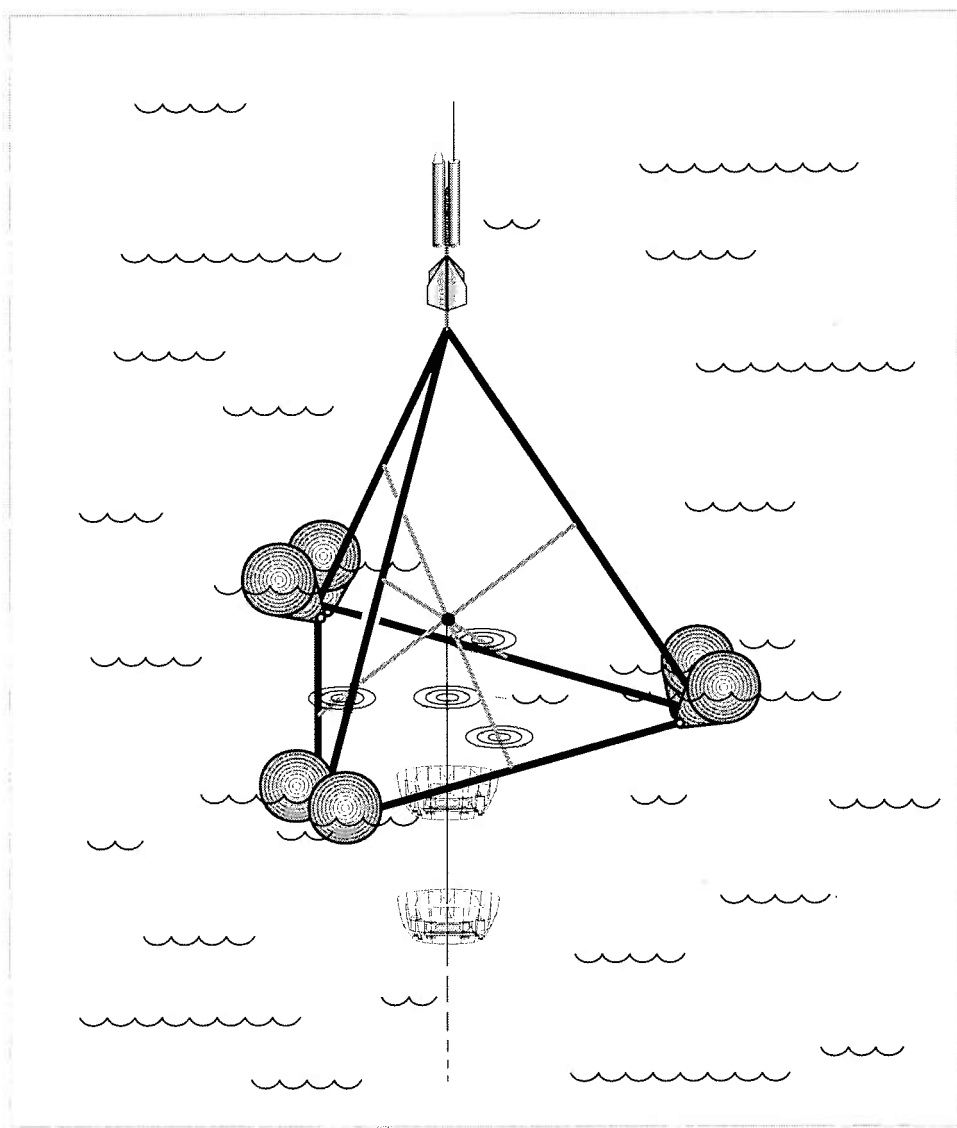


Figure 2.4: Optical Buoy. The optical buoy allows the end-point incubation of samples under *in situ* light and temperature conditions for CO production and CO oxidation rate measurements. The buoy is easily deployable and free-drifts within the water-mass, and maintains samples at discrete and predetermined depths. Deployment occurs at dawn and recovery at dusk, thus the incubation period spans the entire photoperiod. Buoy design by O. Zafiriou.

BATS166 substrate-series.

Sample water was collected by Niskin flask on a Kevlar wire and drawn into an acid-cleaned 2 liter Erlenmeyer flask. The sample water was sparged for 30 minutes with zero-grade air passed through a heated CuO catalyst designed to oxidize all environmental CO, allowing a tightly constrained specific activity of the label to be used in the assay. Stock label was produced by acid dehydration of formate (as previously described) in 100 μCi batches in 120 ml serum vials. The labeled product was transferred to an evacuated Tedlar[®] gas sample bag by displacement with CO-free distilled water injected into the 120 ml vial, connected to the Tedlar[®] bag with 2 mm Teflon[®] tubing with 22g hypodermic needles attached at either end. The flexible Tedlar[®] container removed the need to monitor volume/pressure effects with gas removal, as was required with rigid glass stock bottles. 25.0 ml of sparged (CO-free) sample water was pipetted into 70 ml serum vials that had been flushed with CO-free air for 30 seconds at a flow rate of 5.0 ml sec⁻¹. Headspaces were continuously flushed with CO-free air until each vial was sealed. Labeled CO was injected with a gas-tight syringe into each vial set. The dissolved [CO] in different incubation sets increased incrementally from low (0.095 nM dissolved CO from 20 μl injected) to high [CO] (9.493 nM from 1000 μl injected).

RESULTS and DISCUSSION

COASTAL CO STUDIES: Vineyard Sound, MA

CO consumption measurement comparison.

Water collected from Vineyard Sound in March, April, and June 2001 underwent a direct comparison of two different CO process measurements, (dark CO consumption in sealed syringe incubations and ^{14}C -CO-oxidation) which are shown to closely agree. Via the equation $[\text{CO}]_t = [\text{CO}]_0 \times e^{k_{co}t}$, the first order rate coefficient k_{co} (hr^{-1}) calculated in the syringe assay was used to calculate CO_2 produced during the incubation. The calculated CO_2 production calculated from k_{co} determined by gas analysis was plotted together with actual ^{14}C - CO_2 during the incubation (figure 2.5). The theoretical rate of CO_2 production based k_{co} from syringe incubations agreed with actual CO_2 production measured in the ^{14}C -CO oxidation assay within 12% in March, 3% in April, and 8% in June. This indicates that most if not all CO disappearing from natural water is being oxidized to CO_2 , and the dominant sink of CO is accounted for.

We confirmed that CO oxidation was biologically mediated by a series of ^{14}C -CO incubations with three separate killed controls (autoclaved, 0.22 μm -filtered, and cyanide) and a live natural sample (figure 2.6). The live sample oxidized CO at a rate of $0.516 \text{ nM CO hr}^{-1}$ ($k_{co} = 0.018 \text{ hr}^{-1}$). The three killed samples showed no measurable CO-oxidation activity after 5 hours of incubation with ^{14}C -CO, and supports the findings of Conrad and Seiler (1980) that azide poisoning, boiling, and filter sterilization abolished CO consumption in seawater. The autoclaved and cyanide treated samples had

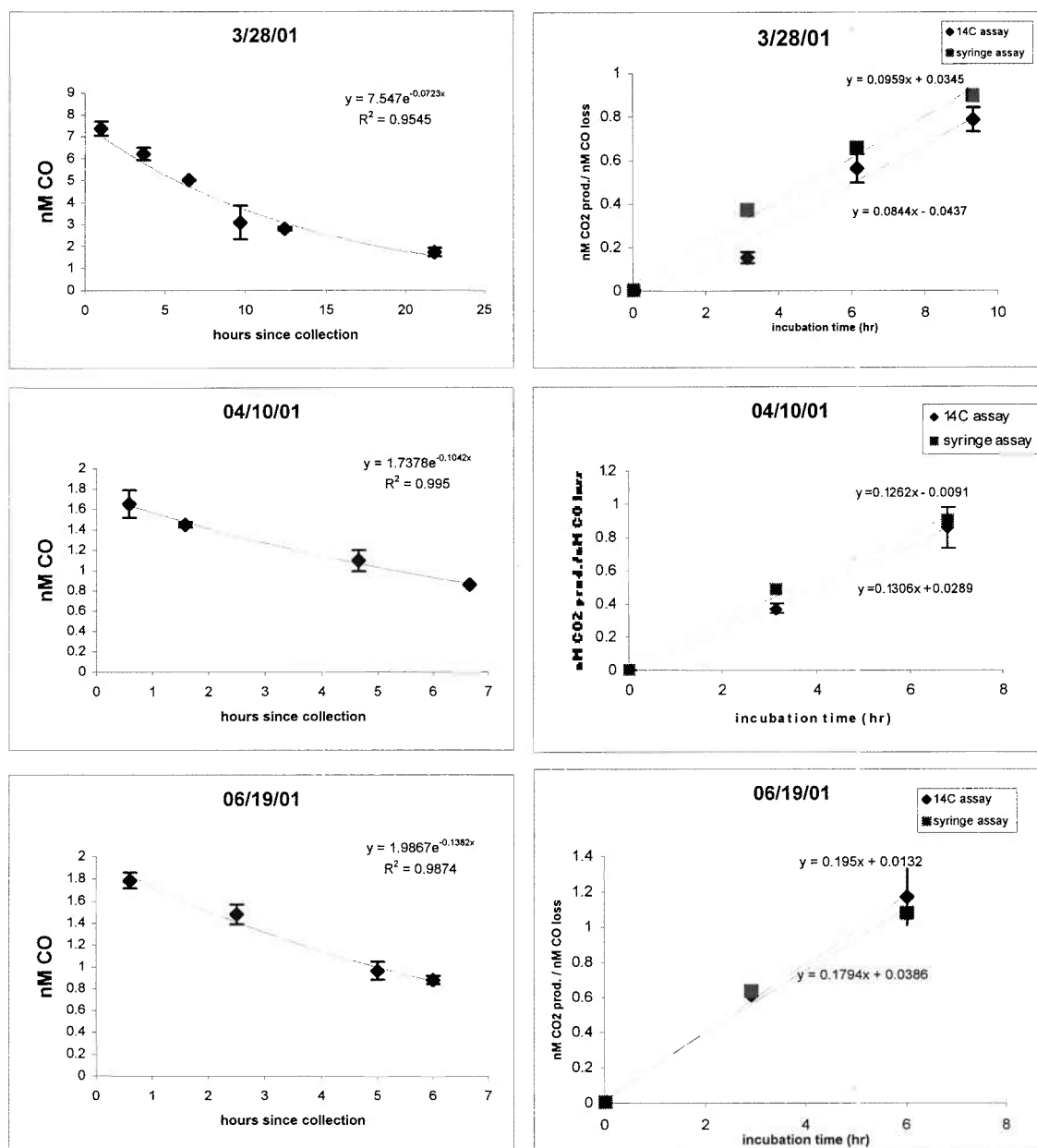


Figure 2.5: Comparison of CO assays. (left column) Syringe assays to monitor CO decay from closed samples result in a determination of rate constant (k_{co}). (right column) Theoretical CO₂ production (■) calculated using k_{co} from syringe assay was directly compared with actual CO₂ production determined by the ¹⁴C-CO oxidation assay (◆).

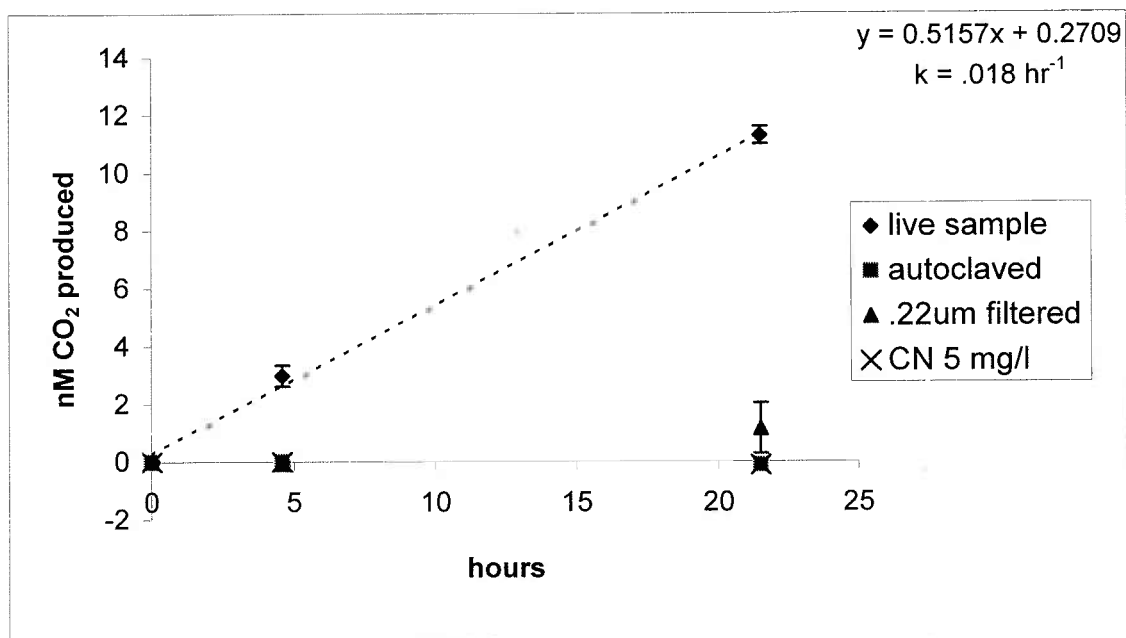


Figure 2.6: CO oxidation is biologically mediated. CO consumption is abolished when variety of sterilization treatments applied to natural seawater samples, compared with the untreated (live) control (◆). (02/20/02)

no measurable activity after 21 hours of ^{14}C -CO incubation, however, there was low level oxidation activity detected in the 0.22 μm filter-sterilized sample after 21 hours, perhaps due to extremely small cells that had passed through the filter, or by filter blow-by into the filtrate.

Compartmentalization of carbon derived from ^{14}C -CO was investigated by time-series measurements of ^{14}C activity in acid-unstable products (^{14}C -CO₂ in headspace gas) and acid-stable ^{14}C -POM and ^{14}C -DOM on filters and in filtrate. Time-series CO consumption within syringe samples (natural seawater) was measured directly with a Trace Analytical reduction gas analyzer. To determine the fraction of CO that is oxidized to CO₂, the ^{14}C -CO-oxidation assay was employed. The fraction of CO-born CO₂ that was incorporated in organic material (acid-stable products) was determined with ^{14}C liquid-scintillation-counting of filtered material (POM) and filtrate (DOM). It was determined that the filters and liquid filtrate from these incubations contained only background levels of activity (<50 DPM) (figure 2.7). As the harsh chemical treatment during sample processing likely destroyed the cells, filter activity was expected to be minimal. Neither the filter nor the filtrate retained any labeled carbon fixed as recalcitrant organic material, and all labeled carbon that had been metabolized by the microbial community was captured quantitatively at the acidification step. A modified ^{14}C -CO oxidation experiment to determine ^{14}C -CO incorporation into particulate material was performed with pure cultures and without the destructive pH treatments, and will be discussed in detail in Chapter 3.

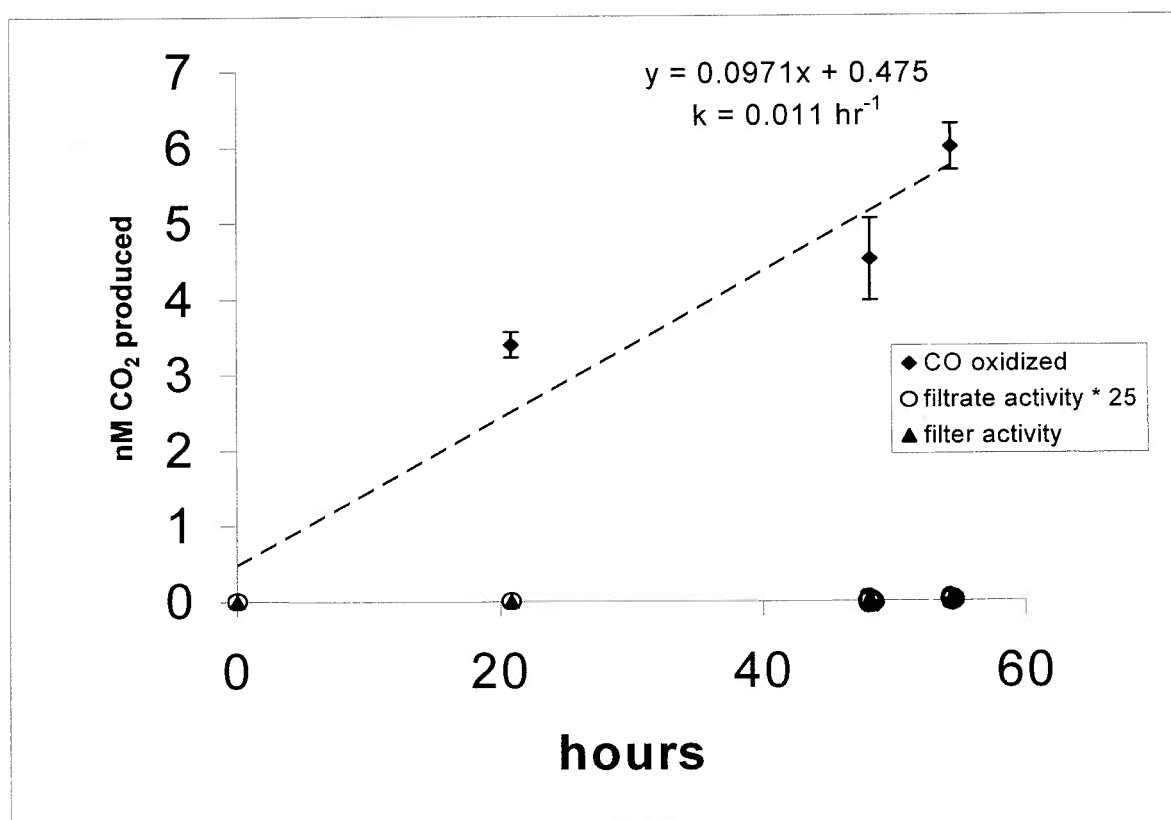


Figure 2.7: CO is oxidized rather than incorporated into particulate matter. In a long-term parallel incubation (01/25/01), CO_2 product and filtered particulates were measured for ^{14}C activity after incubation with ^{14}C -CO. All measured activity was in the form of bicarbonate or other acid-volatile products.

Reduced gas analysis (Trace Analytical RGA) of *in situ* [CO] and rates of CO oxidation (figure 2.5) at the coastal sampling location are consistent with other coastal CO oxidation measurements in similar environments (*Jones & Amador, 1993; Jones, 1991*). CO production is typically greater near coastal waters than open-ocean sites, probably due to larger CDOM content. The mid-day peak of CO concentration (figure 2.8), for example, is 3 – 5 fold higher than the maximum measured in the Sargasso Sea (*Jones, 1991; Xie et al., 2002b*)(figure 2.14). CO-oxidation rates in coastal waters are approximately an order of magnitude greater than those measured in oligotrophic environments, suggesting an active CO-oxidizing microbial community near-shore.

In a study to determine kinetic parameters in coastal water samples in December 2000, we estimated V_{\max} and half-saturation constant (K_M) by graphic determination (figure 2.9) and by Lineweaver-Burke calculation (figure 2.10). The approximate K_M of the coastal assemblage for CO is 1.55 nM CO, and is in agreement with the approximate K_M for the oceanic assemblage (figure 2.18).

The CO-oxidation rate constants measured at the coastal sampling location are highly variable on a seasonal or annual scale (figure 2.12). The mean value for all coastal measurement of k is $0.064 \pm 0.021 \text{ hr}^{-1}$ ($n=9$, mean \pm SEM). The mean coastal value for k_{co} is 6-fold greater than the mean oceanic value for k_{co} during the winter EN327 cruise, and ~2.8-fold greater than the summer EN335 value. This greater rate of CO oxidation in coastal waters reflects either a faster specific CO-oxidation activity on a per-cell basis (figure 2.13), a higher cell density (figure 2.11), or both. Per-cell specific activity for CO oxidation was calculated for the coastal assemblage with CO oxidation rates and AODC

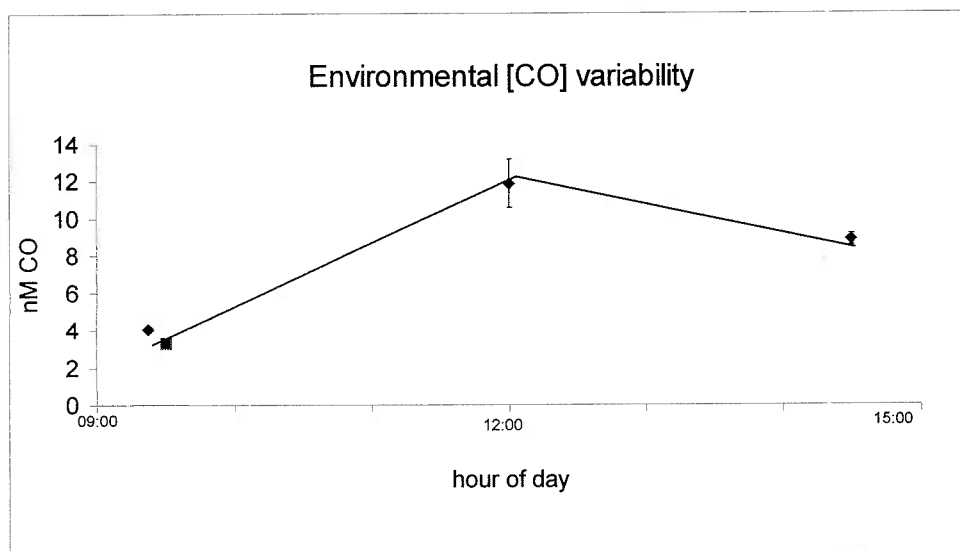


Figure 2.8: Diel [CO] variability was measured with a reduced-gas analyzer. *In situ* [CO] was determined at three sampling times on 10/20/00.

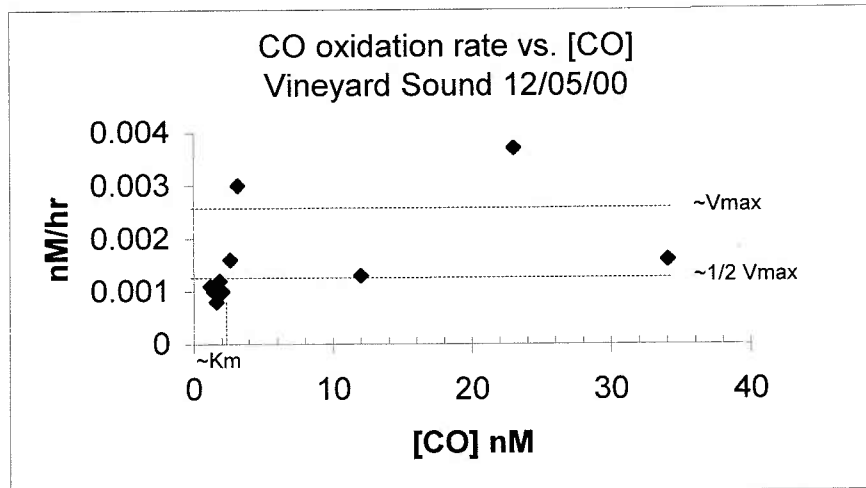
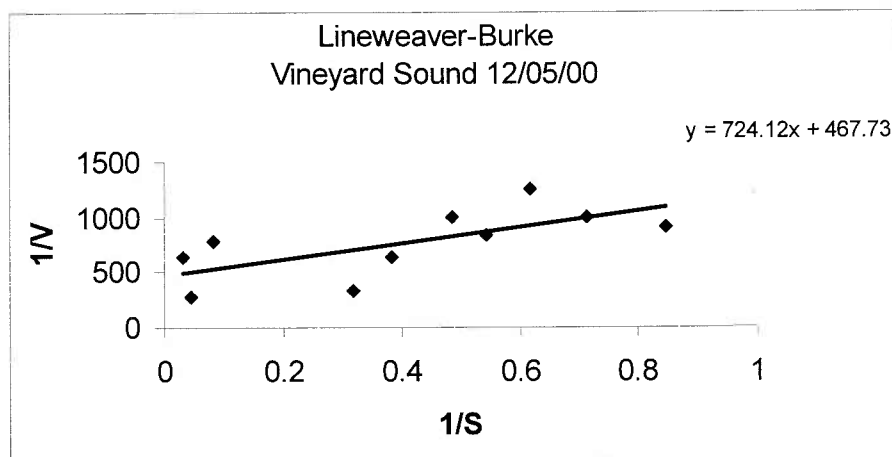


Figure 2.9: Kinetics experiment to estimate V_{\max} and K_M for coastal microbial assemblage for CO. Dissolved [CO] results from injected tracer only.



$m =$ 724.12
 $b =$ 467.73
 $V_{\max} =$ 0.0021
 $X_{\text{int}} =$ -0.645
 $K_M =$ 1.548

Figure 2.10: Lineweaver-Burke calculation to estimate K_M for CO in a coastal water sample.

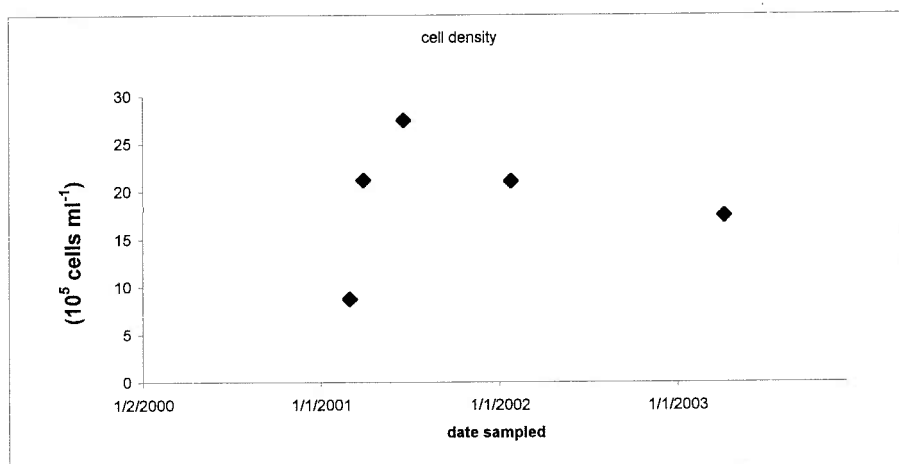


Figure 2.11: Cell density over time at Vineyard Sound, MA

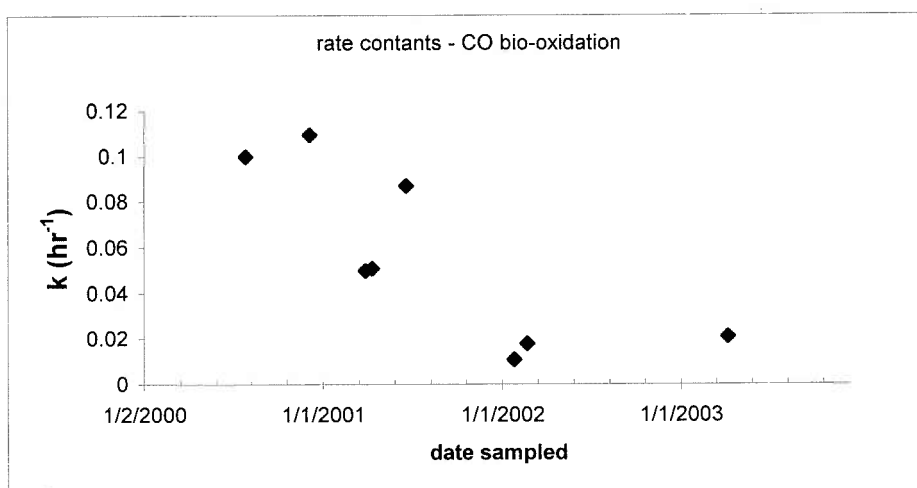


Figure 2.12: Rate constants (k_{CO}) measured at the coastal sampling locations at several times over three years.

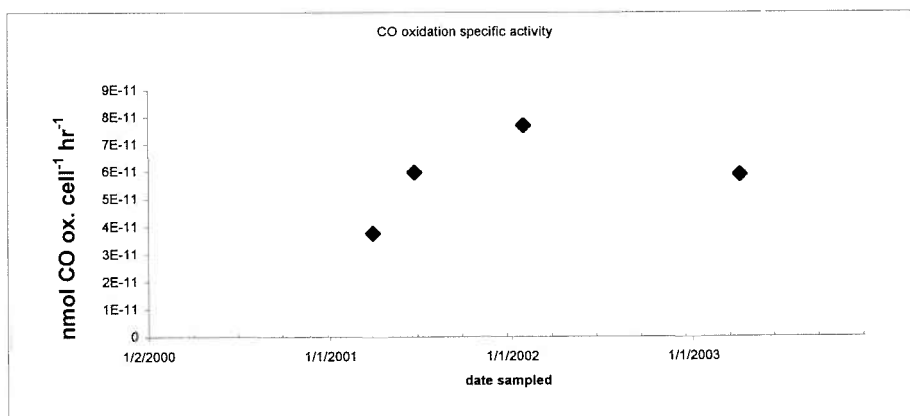


Figure 2.13: CO oxidation per-cell specific activities at the coastal sampling location, calculated from CO oxidation activity and *in situ* cell density at various sampling dates.

data, and is in the range of $4.5 \times 10^{-11} - 6 \times 10^{-11}$ nmol CO oxidized cell⁻¹ hr⁻¹. This value should be regarded as the minimum value of activity by environmental isolates to designate them as environmentally important CO oxidizing strains, since this calculation assumes that every cell in total assemblage oxidizes CO at the same rate. Organisms of interest should have at least this rate of cell-specific activity.

Since dissolved [CO], biological CO oxidation rates and rate constants, cell density, and per-cell specific CO oxidation activities are all several-fold to an order of magnitude greater in a coastal environment compared with an oligotrophic environment, the coastal waters are therefore a promising source of CO-metabolizing microbial isolates, to be explored in Chapter 3.

Sargasso Sea CO bio-oxidation studies:

Six *in situ* optical buoy incubations were performed in collaboration with O. Zafiriou (WHOI Dept. Chemistry) over an eight-day period in August, 1999 (EN327), and seven over an eight-day period in March, 2000 (EN335). Each buoy was deployed at dawn and recovered at dusk. Sample water was collected from a single depth (20 m) early in the morning (0100 – 0300) for dispensation and incubated at five to seven discrete depths with end-points spanning the integrated photic period. Additionally, dark time-series rate measurements were performed with samples collected via Niskin bottles at various depths and at times around the clock to compare with the *in situ* measurements to establish whether *in situ* light intensity may inhibit biological CO oxidation. Cruise data were divided into four groups: summer buoy (*in situ*), summer dark, spring buoy,

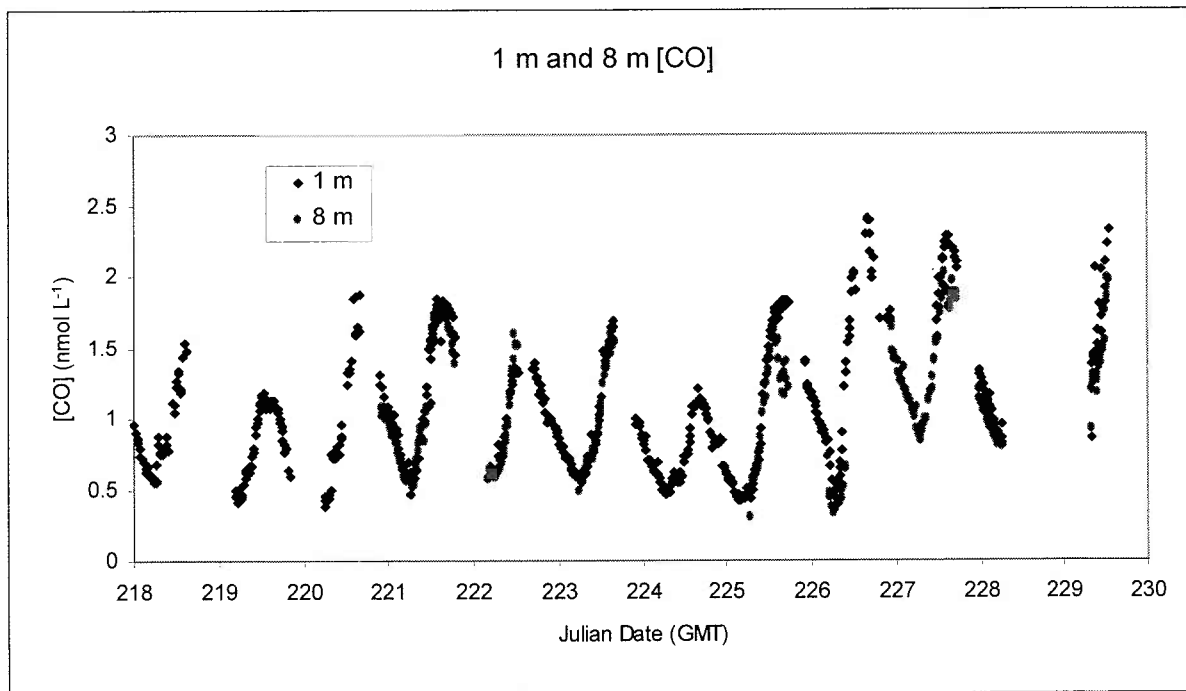
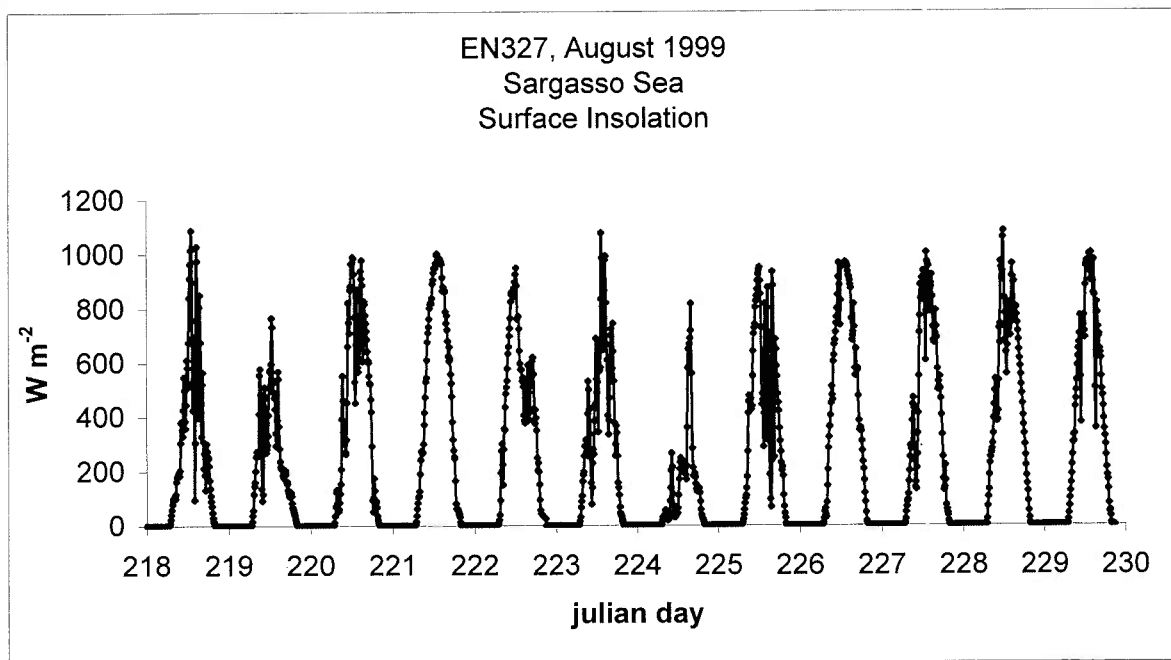


Figure 2.14: Diel monitoring of dissolved [CO] in oceanic (BATS) surface waters. Top: Daily solar intensity, sampling interval 15m. Bottom: dissolved [CO] at 1m (◆) and 8m (●) depth (from Xie *et al.*, 2001, 2002a, 2002b).

and spring dark. Initially, rate coefficients (k_{co}) were determined by normalizing the observed rates by the concentration of total dissolved [CO] within the sample. Dissolved [CO] varied in samples of a given cruise because of differences in *in situ* [CO] depending on the time of day sampled, and differences in injected ^{14}C activity that arose because of the fixed-volume stock vessels. In principle, if first order rate kinetics are in effect, then the average rate coefficient k_{co} should remain stable with increasing [CO]. However, when all dark rate coefficients were plotted versus [CO], we observed a trend of decreasing rate coefficients with increasing dissolved CO in both cruises (figure 2.15). At some level of dissolved [CO], first order rate kinetics is no longer followed and zero order (saturation) kinetics takes effect. The microbial assemblage in the surface Sargasso Sea is typically subjected to low CO concentrations with maximum exposures of $\sim 2 - 2.5$ nM CO for a brief period during mid-afternoon during peak photoproduction. The injection of labeled CO to each sample vial was sufficient to raise the dissolved [CO] within the sample to exceed environmental levels (figure 2.17) for an extended period of time (12-14 hours). This long period of higher than- environmental CO levels may cause inhibition of CO oxidation of the microbial assemblage, and may account for the depressed rate coefficients observed in many of our measurements. More likely, however, is the CO oxidizing enzymes of the active microorganisms became saturated at high [CO], and normalization of the rate in obeisance to first order kinetics resulted in an underestimate of the rate coefficient in incubations containing [CO] higher than the saturation level.

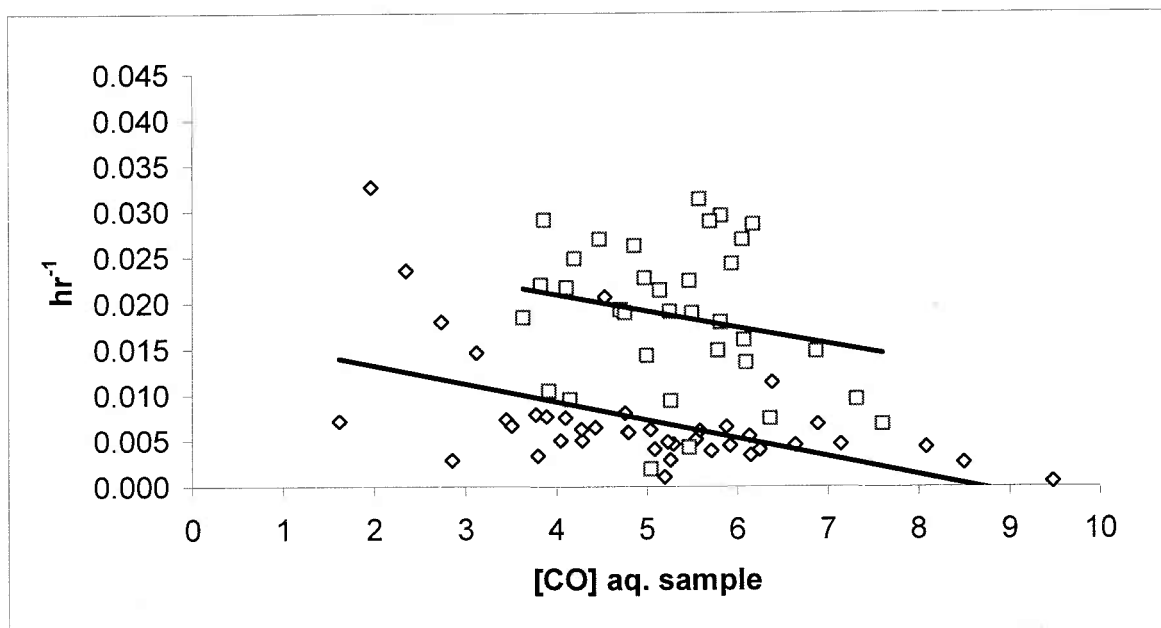


Figure 2.15: Empirically derived rate coefficients from all incubations during EN327 (\square) (summer) and EN335 (\diamond) (spring) suggest rate saturation at higher $[\text{CO}]$.

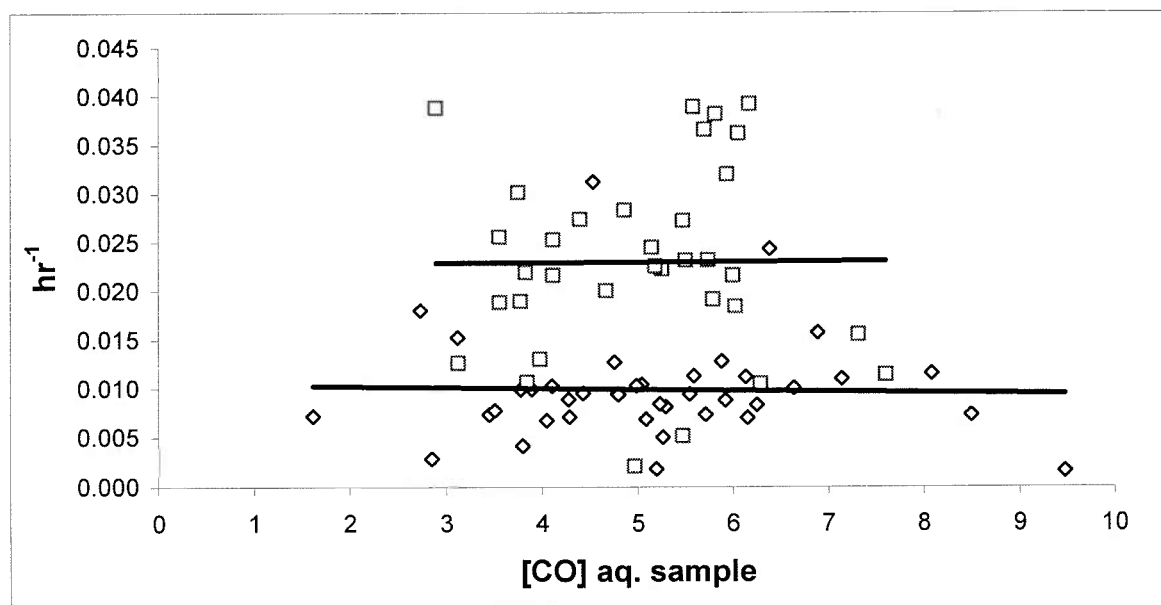


Figure 2.16: Depressed rate coefficients from high- $[\text{CO}]$ incubations corrected by normalization by inhibitory $[\text{CO}]$ value; 4.5 $n\text{M}$ during EN327 (\square) and 3.5 $n\text{M}$ during EN335 (\diamond). Average corrected rate coefficients (k_{co}) for EN327 = 0.023 hr^{-1} , and EN335 = 0.010 hr^{-1} .

In the dataset for each cruise, we inferred the saturating [CO] levels by applying a fixed normalization factor (CO concentration) in incubations where the dissolved [CO] was higher than peak environmental levels. In incubations having total dissolved CO below the saturation level, each rate constant was calculated simply by normalizing the measured rate (nM/hr) by the total dissolved [CO] (nM) within the incubation set, obeying first order kinetics. In incubations containing dissolved [CO] greater than the inferred saturation concentration, rate constants were normalized by the single [CO] value where first order kinetics gives way to zero order. These transition points were determined by observing where the average value of k_{co} remained constant across all [CO] (figure 2.16), and were 4.5 nM CO during EN327 and 3.5 nM CO during EN335. The average rate constant for CO oxidation after this correction was applied to the data was $0.023 \pm 0.0017 \text{ hr}^{-1}$ ((mean \pm SEM) in the summer cruise (EN327), and $0.010 \pm 0.00090 \text{ hr}^{-1}$ in the early spring (EN335).

We validated this correction approach to apply to the 1999 and 2000 rate data in a substrate-series experiment conducted in July 2002 aboard the R/V *Weatherbird* (Bermuda Biological Station for Research, Inc.) in the vicinity of the BATS site (BATS166) and the previous EN cruises. A careful and systematic series of time of series incubations under aqueous CO concentrations ranging between 0.01 and 10 nM were performed (0.1 nM-intervals between 0 – 1.0 nM, and approximately 1 nM-intervals between 1.0 and 10 nM) to establish the [CO] where saturation of CO oxidation occurs (Figure 2.17). The time-series consisted of paired samples sacrificed approximately 2 hours apart in 8-hour incubations. Incubations and processing of samples were

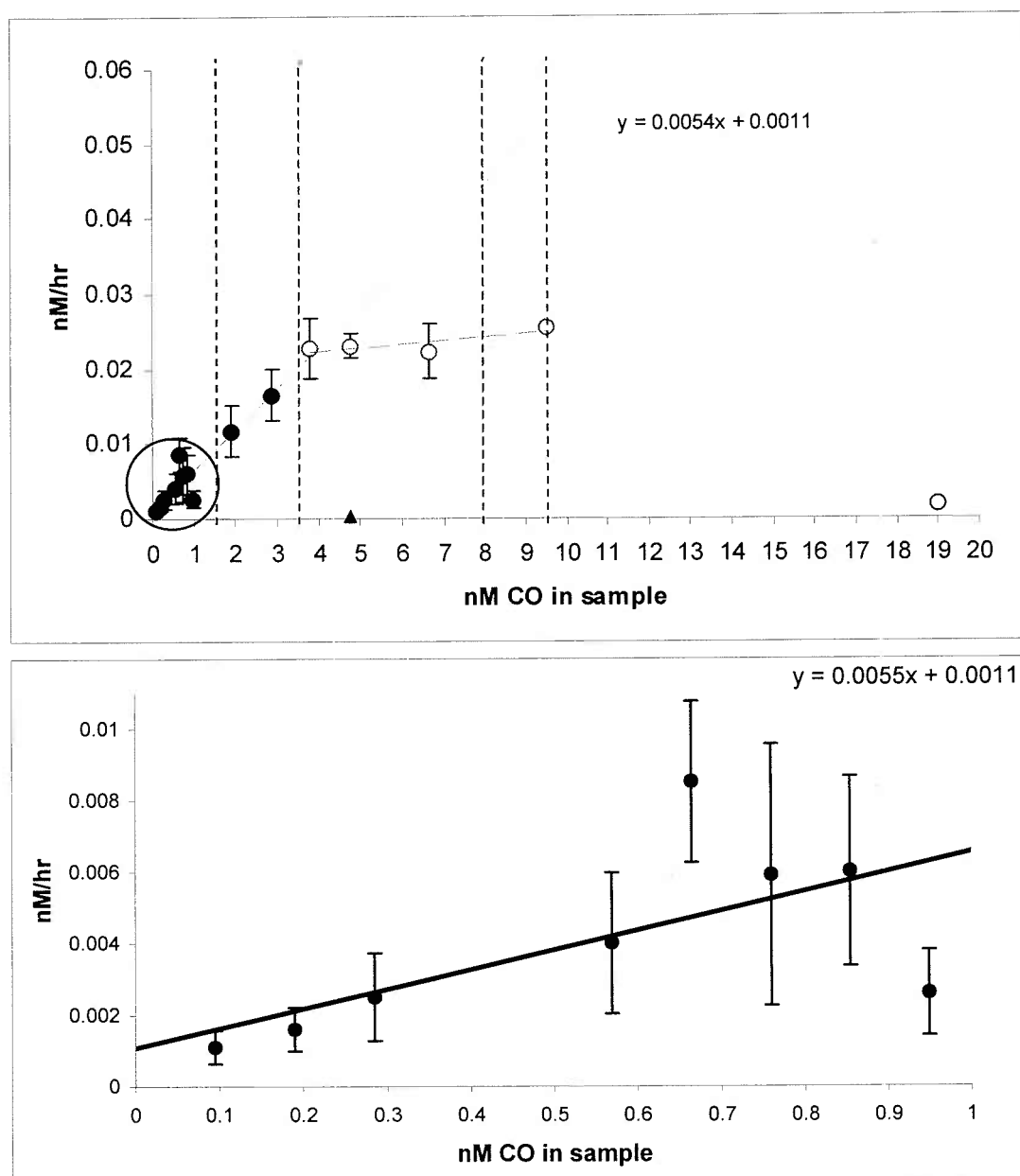


Figure 2.17: Substrate series performed on microbial samples near BATS, July 2002.

(upper panel) Area between outer dashed vertical bars is the range of dissolved [CO] in incubations performed during EN335, and during EN327 between inner dashed vertical bars. Closed circles (●) designate the region obeying first-order kinetics. Open circles (○) designate the inhibitory region or deviation from first-order kinetics. Black triangle (▲) is a killed control (CN).
 (lower panel) Linear relationship of rate and dissolved [CO] at [CO] less than 1 nM (region encircled in top panel is expanded in lower panel)

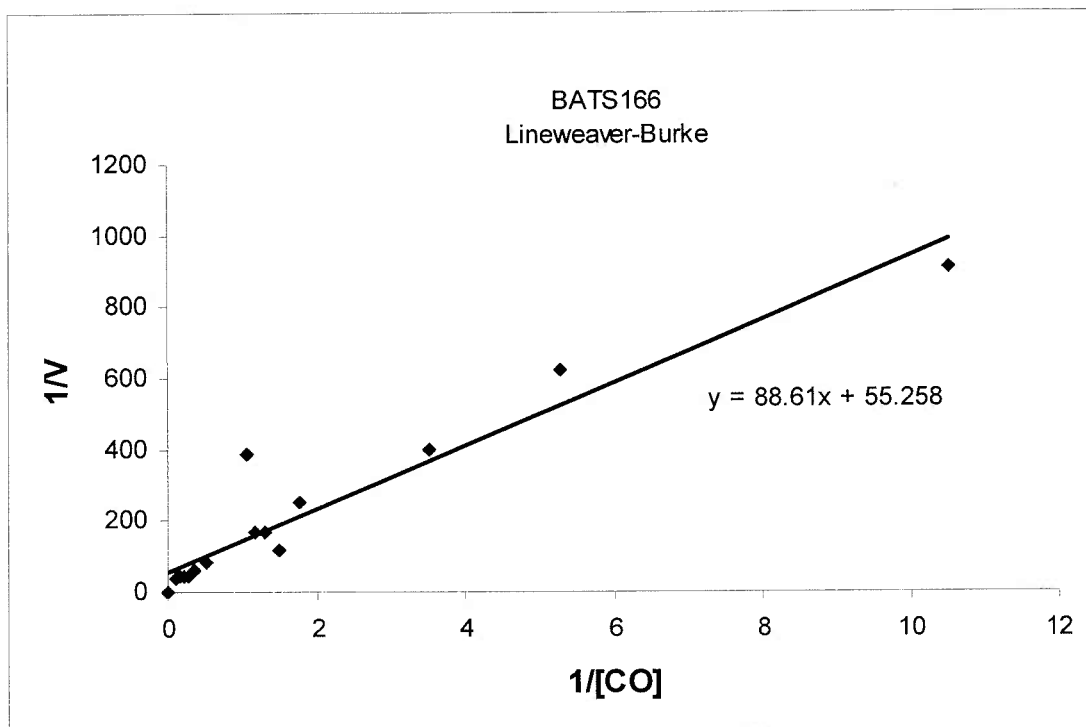


Figure 2.18: Kinetic analysis of BATS 166 substrate series. $V_{\max} = (1/b) = 0.020$ nM/hr.
 $K_m = (-1/X \text{ intercept}) = 1.793$ nM CO.

performed as previously described. Lineweaver-Burke analysis of the substrate series experiment showed a maximum velocity for the CO oxidation reaction (V_{\max}) of 0.020 nM CO oxidized hr^{-1} and the apparent half-saturation constant (K_M) was 1.793 nM CO (figure 2.18). We observed deviation from the straight-line typically observed in the analytical procedure used to calculate K_M and V_{\max} in incubations having dissolved CO concentrations greater than 4.0 nM in the BATS166 experiment, which is bracketed within the two saturation [CO] concentrations inferred from the EN327 and EN335 cruise data. Incubations containing greater than 4 nM dissolved CO did not result in increasing CO oxidation rates. CO oxidation activity was nearly abolished in an incubation containing 19 nM CO, or six-fold greater [CO] than experienced *in situ* ($4 \times 10^{-4} hr^{-1}$). This suggests that saturation kinetics gives way to inhibition kinetics at [CO] between 10 nM and 19 nM and possibly at even lower [CO]. There are no data to interpolate between these two [CO] values in the BATS166 cruise because our objective was to determine the transition point between first and zero-order kinetics at low [CO], therefore the transition between CO saturation and inhibition is poorly constrained.

When the EN cruise data were corrected for substrate saturation effects due to high [CO] in ^{14}C -CO incubations, the highest observed CO oxidation rates were observed during the summer 1999 cruise where the dark incubation rate constant ranged between 0.019 to 0.028 hr^{-1} (figure 2.19) with the maximum occurring at 15m - 20m. These rates were ~10-fold higher than the *in situ* rates for that season (0.0017 – 0.0025 hr^{-1}), and also higher than both the dark incubation and *in situ* rates for the spring season (figure 2.20), which ranged between 0.0083 - 0.017 hr^{-1} and 0.0041 - 0.0062 hr^{-1} ,

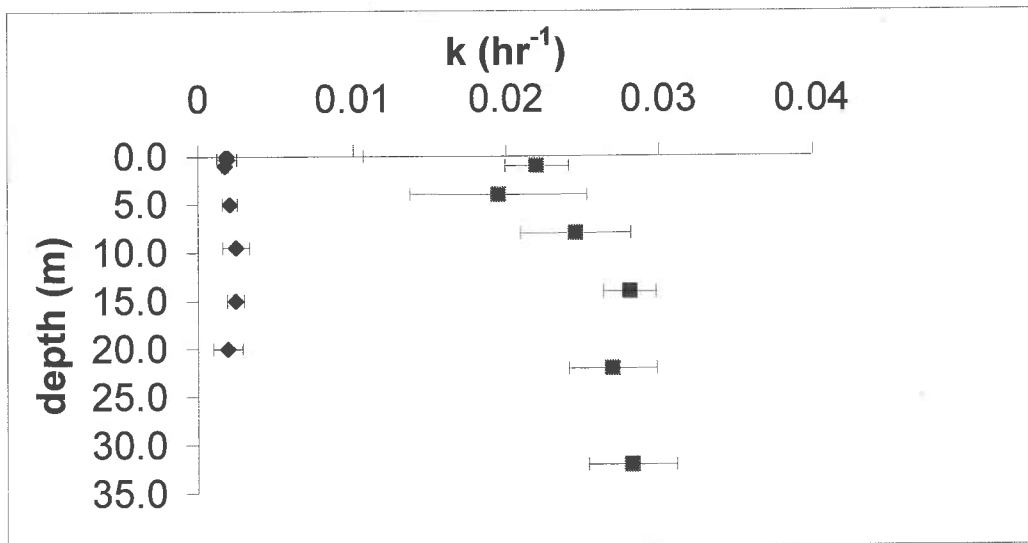


Figure 2.19: EN327 (summer) buoy (♦) and dark incubation (■) rate coefficients.

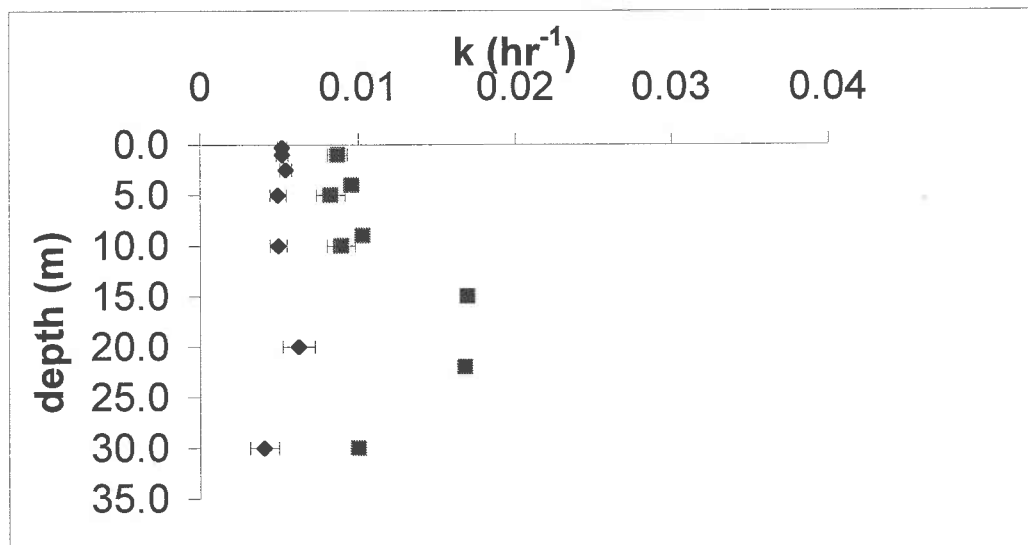


Figure 2.20: EN335 (spring) buoy (♦) and dark incubation (■) rate coefficients

respectively. We did not observe increasing CO oxidation rates with increasing depth in any *in situ* incubations. The *in situ* CO-oxidation rates are depressed relative to the dark incubations during both cruises. The light field at all *in situ* incubation depths may have been inhibitory to CO oxidation by the microbial assemblage, and thus depressed the CO-oxidation rates at all *in situ* depths tested (0-30m) relative to dark incubations at corresponding depths. This suggests that daytime dissolved [CO] increases rapidly from the combined effects of photoproduction and light-induced inhibition of the microorganisms that consume CO. Similarly, nighttime dissolved [CO] decreases rapidly when photoproduction ceases and activity of CO metabolizing microorganisms resumes.

Specific CO-oxidation activities for the spring and summer seasons were estimated using the CO-oxidation rate constants measured in this study and the bacterioplankton standing stock estimates of Carlson *et al.*, (1996). Their estimates were 4×10^8 cells l⁻¹ in spring and 5×10^8 cells l⁻¹ in summer in the surface mixed layer. The summer and spring mean cell-specific CO oxidation rates were $1.3 \times 10^{-11} \pm 2.9 \times 10^{-12}$ nmol CO oxidized cell⁻¹ hr⁻¹ and $2.8 \times 10^{-11} \pm 3.2 \times 10^{-12}$ nmol CO oxidized cell⁻¹ hr⁻¹, respectively. These activities are calculated using our measured values for total CO oxidation rate and literature values for bacterial cell density, and represent a minimum value of per-cell CO oxidation activity and environmental relevance since not all cells oxidize CO.

Our estimates of oceanic and coastal CO consumption rates in the surface water are consistent with prior studies that have investigated this parameter with syringe assays (Conrad & Seiler, 1982), ¹⁴C-CO oxidation methods (Jones & Amador, 1993; Jones,

1991) or by calculation using numerical models (Johnson & Bates, 1996). Conrad *et al.* (1982) and Conrad & Seiler (1982) measured the decay of CO in surface samples from the equatorial Atlantic Ocean, and reported rate constants on the order of $0.03 - 0.05 \text{ hr}^{-1}$ in dark incubations, and K_m values for CO to be 7 nM CO , only slightly higher than our estimates for these parameters in oceanic waters. Jones (1991), using the ^{14}C -CO oxidation method in dark incubations, measured rate coefficients for CO consumption in the Sargasso Sea surface waters that were $0.012 - 0.018 \text{ hr}^{-1}$ in July, and $0.0045 - 0.011 \text{ hr}^{-1}$ in September. Jones and Amador, (1993), in a study to determine the impact of the Orinoco River on the consumption of CO and CH_4 and the photoproduction of CO in the southeastern Caribbean Sea, reported CO oxidation rates were minimum at a station in Mona Passage (0.028 hr^{-1}), increased with proximity to the Orinoco River outflow, and were highest near the mouth of the river (0.199 hr^{-1}). Johnson & Bates (1996) estimated CO loss rate constants by an exponential fit method, and reported mean k_{co} values as $0.032 \pm 0.0071 \text{ hr}^{-1}$ in April, and $0.009 \pm 0.0052 \text{ hr}^{-1}$ in December. Rate estimates in our study, in both oceanic and coastal waters, nicely fit with CO loss rates previously determined by a variety of methods. One assumption common to the aforementioned studies, however, is that the CO oxidation rate at a given station does not vary between day and night; all direct measurements of CO consumption rates have thus been made with dark incubations. Our daytime *in situ* incubations indicate that there may be a light-related inhibition of CO oxidation. CO turnover times commonly reported in “days” (the reciprocal of the rate constant in terms of “ day^{-1} ”) may not be accurate if *in situ* CO oxidation is retarded in daytime light regimes. If CO oxidation is slower during daylight

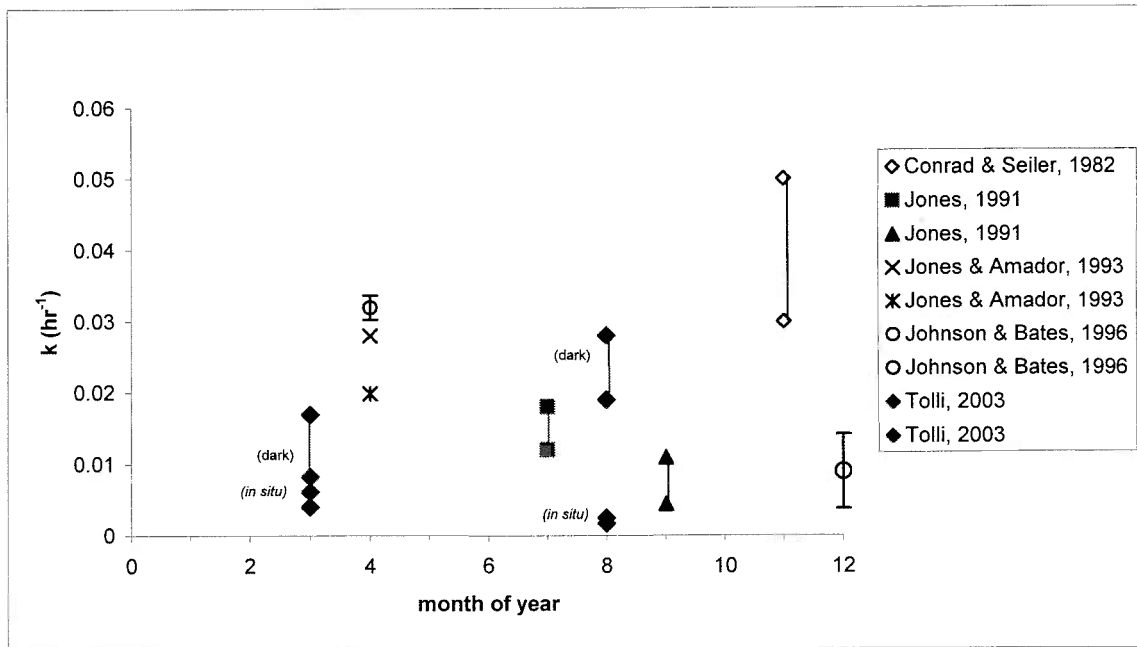


Figure 2.21 Comparison of oceanic rate coefficients (k_{co}) measured in this study (◆) with literature values. Literature values of k_{co} (hr^{-1}) were calculated from values reported as “day⁻¹” or the reciprocal, “days” (turnover time). All literature values of oceanic k_{co} are based on dark incubations, and do not account for potential daytime rate inhibition.

hours, then the diel rate coefficients k_{co} are high and CO turnover times reported previously are underestimated (figure 2.21). Turnover time calculated using our summertime dark incubation values integrated over 24 hours is 1.67 days. If daytime rates are inhibited by an order of magnitude over the photic period (12 hours), then the integrated turnover time (12 hours daytime, 12 hours dark) is 3.07 days, or 83% higher.

Prior studies of CO processes in oceanic and coastal waters, while lending support to our results, still do not provide insight to the microbial species that are responsible for the observed CO oxidation. The phylogeny and identity of cells responsible for CO oxidation in the Sargasso Sea remains enigmatic; Chapters 3 and 4 of this study are concerned with the identities of coastal CO oxidizing organisms, where organisms belonging to the genus *Roseobacter* and *Paracoccus* were found to be important CO oxidizers. Giovannoni *et al.* (1990), using PCR-assisted sequence retrieval and fairly general bacterial primers, retrieved partial 16S-rRNA sequences that are affiliated to the *alphaproteobacteria* from the Atlantic Sargasso Sea, among many others. Sequences of the so-called SAR83 cluster had only a few percent difference to those of members of the *Roseobacter* group (Amann, 2000, for review) that were found to be active CO oxidizers in this study. The metabolic importance of *Roseobacter* and other marine-alpha organisms in CO oxidation will be discussed in subsequent chapters.

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CHAPTER III:

Characterization of Marine Carbon Monoxide Oxidizing Microorganisms Isolated from a Coastal Marine Environment

ABSTRACT

For the purpose of isolating a specific phylogenetic group of microorganisms in natural seawater and determining their relative contribution to a gross metabolic feature found in seawater (CO oxidation), we utilize a direct isolation method for obtaining CO oxidizing microbes under conditions approximating the natural environment. The method involves cultivation of bacteria on membrane filters placed atop an oligotrophic liquid mineral medium, subsequent incubation with radiolabeled CO at near-environmental levels, and the use of macroautoradiography to screen colonies with the desired phenotype amidst a potential background of other colonies. Since this method spatially separates otherwise competing populations, simultaneous recovery of organisms with different growth rates and nutritional requirements is possible, and large numbers of bacteria can be screened rapidly at one time. Cell-specific CO-oxidation activities were determined for selected strains with time-series ^{14}CO -oxidation rate measurements. Nine of 30 resulting isolates oxidize CO at environmentally relevant rates, and were taxonomically diverse with representatives in the α , β , and γ -subclasses of the *Proteobacteria*, and in the *Cytophaga-Flavobacterium-Bacteroides* group. Three isolates with the highest specific rates of CO metabolism ($1.0 \times 10^{-10} - 2.0 \times 10^{-10}$ nmol CO cell $^{-1}$ hr $^{-1}$) cluster within the *Roseobacter* and *Paracoccus* groups of the α -*Proteobacteria*, collectively known as the "marine alpha group". CO oxidizing isolates resulting from this study are not related to bacterial groups previously implicated in environmental CO oxidation, rather, they represent a hitherto unreported metabolic function in several diverse microbial types.

INTRODUCTION

Selective liquid culture enrichment techniques have been used to isolate bacteria to study their microbial ecology and physiology. These techniques typically involve incubation of mixed populations in a medium designed to foster the growth of those microorganisms exhibiting a particular phenotype. Serial transfers of the enrichment culture to fresh medium are continued until the microbial culture appears to consist mainly of one or very few types of microorganisms exhibiting the desired properties.

Undesired organisms are eliminated from the culture through competitive exclusion or dilution, or streaking on selective agar media. Although selective enrichment techniques have historically been used with great success, the diversity of organisms in enrichment cultures is not likely to reflect that found in the natural environment. Dominant organism(s) in the enrichment culture may or may not be important players in the natural environment.

Enrichment and isolation of pure cultures of “carboxydophilic” bacteria, microorganisms that are capable of gaining both energy and carbon from carbon monoxide, from natural habitats have traditionally employed liquid batch cultures incubated aerobically in desiccators supplied with CO₂ and high levels of CO as carbon and energy sources. Such conditions are highly specific for the development of carboxydophiles because of the inability of the contaminating microflora to use CO for growth, and binding of CO to the terminal respiratory oxidases of most organisms results in inhibition of their growth (*Meyer & Schlegel, 1983*). Carboxydophilic bacteria possess specialized metabolic machinery for the oxidation of CO, and attempts to isolate them from marine environments (other than hydrothermal sites) are conspicuously absent in the primary marine literature.

In enrichment programs using very high CO concentrations, the resulting isolates generally have a low affinity for that substrate. The reported K_M for CO of carboxydophilic isolates obtained by these methods from terrestrial enrichments containing >500 nM CO (*Conrad et al., 1981; Meyer, 1985*) have been 1-2 orders of magnitude higher than the average K_m for CO consumption in soil or water (5-50 nM)

(Conrad & Seiler, 1982b, Conrad *et al.*, 1981; Bartholomew & Alexander, 1981; Jones & Morita, 1983), or marine environments (1-2 nM) (this study). The observed high K_M for CO by several terrestrial carboxydotrophic strains is likely a bias created by the high [CO] used in the enrichments and for their isolation (Zavarzin & Nozhevnikova, 1977). This highlights the importance of cultivating CO metabolizing bacteria under CO concentrations close to that encountered *in situ* for obtaining microbes that are more representative of those active in the environment.

To this end, we utilized a method that does not rely on the use of liquid batch-culture techniques for screening and isolating microorganisms with particular phenotypes. The method involves cultivation of bacteria on membrane filters atop a combusted glass-fiber filter saturated with an oligotrophic liquid mineral medium, subsequent incubation with radiolabelled CO, and the use of autoradiography to identify colonies with desired phenotypes amidst a potential background of other colonies. Since this method spatially separates otherwise competing populations, simultaneous recovery of organisms with different growth rates and nutritional requirements is possible, and large numbers of bacteria can be screened rapidly at one time. The defined mineral media (Appendix A) used in this study supports growth of carboxydotrophic bacteria and other microorganisms able to metabolize CO, and selects against diverse heterotrophs that cannot survive elevated [CO] or cannot utilize CO for growth. Cell-specific rates of CO oxidation from ^{14}C -CO oxidation studies of selected pure isolates are used to estimate the CO oxidation that is attributable to these organisms *in situ*. We attempt to show that these organisms' apparent abundance and metabolic activity is sufficient to account for a

significant fraction of CO bio-oxidation in natural samples that is measured independently by time-series gas analysis and ^{14}C -CO oxidation techniques.

CO production is typically greater near coastal waters than open-ocean sites, probably due to greater colored dissolved organic matter (CDOM) content (*Jones & Amador, 1993*). The mid-day CO concentration (12 nM) at our coastal sampling location (figure 2.8), for example, is 3 – 5 fold higher than the maximum measured in the Sargasso Sea (*Jones, 1991; Xie et al., 2001*). CO oxidation rates in coastal waters (0.01 – 0.11 hr⁻¹, this study, fig. 2.12) can be up to an order of magnitude greater than those measured in oligotrophic environments, suggesting an active CO oxidizing microbial community near-shore. The coastal waters are therefore a promising source of CO metabolizing microbial isolates. Our field sampling procedures include i) sampling water for the screening and isolation program in sterile borosilicate glass bottles, ii) sampling water in sterile glass syringes for analysis of CO oxidation rates (reduced gas analyzer and ^{14}C -CO \rightarrow ^{14}C -CO₂ methods), and iii) maintaining *in situ* temperature and salinity for incubation temperatures and calculation of the Bunsen solubility coefficient (β) for CO, necessary for determining the dissolved [CO] when CO is provided in the gas phase at a given mixing ratio.

The phylogenetic relationships of CO oxidizing isolates were determined based on their 16S-rDNA sequences. Strain-specific 16S-rDNA sequence information allows the identification of these organisms and the examination of their phylogenic relationships within the framework of published sequences. Here, we focus on the 16S rRNA-based phylogeny of strains resulting from our cultivation program, and an early

estimation as to whether the apparent abundance and activity of recovered strains are sufficient to support the CO oxidation that is measured by independent means in water that is sampled at the same time and location as the microbial assemblage. The ability to both identify strains and determine phylogenetic relationships that 16S-rRNA sequence information provides, combined with cell-specific CO-oxidation rate information from physiological studies of those organisms in pure cultures, are two component data necessary to determine the relative contributions of CO-metabolizing community members to the total CO-oxidation that is observed in natural waters.

METHODS/MATERIALS

Sampling:

Seawater samples for isolation of microbes and for CO-oxidation measurements were collected from the coastal location described in Chapter 2 (Vineyard Sound, MA) and by the sampling methods described therein.

Isolation program.

Screening of microbial colonies grown from natural seawater by macro-autoradiography at once allows detection and isolation of cells utilizing ^{14}C -CO-born carbon, as well as initial estimates of their abundance. Using the method of Dunbar et al. (1996) modified for our experiment, sampled microbial assemblages were serially diluted (triplicate per dilution level) into defined, sterilized oligotrophic artificial seawater media (see appendix A) and deposited on autoclaved 47-mm diameter, 0.22 μm polycarbonate

membrane filters that have been previously washed with methanol and distilled water to remove potential organic contaminants, and autoclaved. The filter was placed atop a washed and combusted glass-fiber filter saturated with liquid oligotrophic growth medium in a sterile polystyrene petri plate. The growth requirements of known carboxydophilic bacteria are simple; Meyer and Schlegel (1983) recommend an improved trace element solution, which includes molybdenum and selenium for the formation of fully active CODH in carboxydophilic bacteria. To support the growth of lithoautotrophic organisms that may incorporate CO-born carbon only when NH_4^+ is available as an energy source, NH_4^+ is supplied in separate plate sets. Because of the potential for heterotrophic growth on trace vitamin additives to the medium (B_{12} , niacin, and biotin) these vitamins are omitted in duplicate plate sets (preliminary experiments suggest possible need for vitamins at trace levels for some CO metabolizers). In the event that there exists a group of organisms that co-oxidize CO while obtaining their energy requirements from unknown heterotrophic substrates present in natural coastal seawater, a set of membrane incubations were run using natural 0.22 μm filtered coastal seawater from the sampling site as media to supply environmental DOC to this group.

The membranes are incubated in a ^{14}C -CO-containing atmosphere for sufficient time (2-3 weeks) to allow for the induction of enzymes necessary for CO metabolism by the presence of CO and visible colony formation on the membranes. The atmosphere is maintained by injecting the ^{14}C -CO and air mixture into an evacuated dessicator containing the samples. A slight negative pressure maintained within the chamber provided a tight seal. Providing an atmosphere enriched with CO and removing

dissolved organic substances specifies the conditions for the growth of CO metabolizing bacteria that are able to utilize CO as their sole source of carbon and energy, while selecting against CO-sensitive and other heterotrophic organisms. We provided growth conditions for the cultivation of organisms capable of CO metabolism under elevated environmental levels of CO (50 ppmv CO:air = ~ 42 nM CO (aq)).

A series of four sample concentration levels (0.1, 1.0, 10.0, 100.0 ml per membrane) were included in the first incubation. For subsequent sample incubations, we selected the concentration level (undiluted, 1.0 ml per membrane) that resulted in membrane colony densities that were sufficient for statistical analysis and appropriate to macro-autoradiography and replicate plating. Further refinement of the experimental treatment regime included a reduction of different [CO] atmospheres from three concentration levels to only one, dispensing with the high CO atmospheres after one cultivation round when no visible colonies resulted.

Macro-autoradiographic screening protocol.

After the master filters had incubated and supported visible colonies, replicas of the masters were made. First, the master was removed from its media and placed colony-side-up on a sterile square of Whatman #1 paper (figure 3.1). Then, a sterile, wet 0.22 μ m nucleopore filter was placed on top of the master filter, covered with another square of Whatman filter paper, and evenly compressed with a surface-sterilized Plexiglas weight. Reference notches were cut on the stacked master and duplicate filters with a sterile razorblade to record the orientation of the two filters relative to one-another.

Following duplication, the filters were carefully separated and each was placed cell-side-up on fresh mineral media and re-grown for several days in a ^{14}C -CO-containing atmosphere. Following the second growth period, the duplicate filters were removed from the supporting media and allowed to air-dry. When dry, the duplicate filters were spotted with $\text{NaH}^{14}\text{C-CO}_3$ tracer with a sterile toothpick at the location of the registration notches. The dry duplicate filters were attached to 8" x 10" sheets of absorbent Bench-Kote paper with small pieces of transparent tape at the edges of the filter, sample-side up. In a photographic darkroom, an autoradiography cassette was loaded in the following order: filter page within a Kodak BioMax LE (low energy) intensifying screen, followed by Kodak XAR paper placed on top of the intensifying screen. The cassette assembly was closed and allowed to expose for 5-7 days at -80°C . The film was developed under darkroom conditions following standard procedure for X-ray film (3 min. developer, 30 sec. rinse, 3 min. fixer, 20 min. wash). The ^{14}C -label-spotted orientation notches visible on the autoradiograph (figure 3.3) were used to orient the master filters with the developed autoradiographic images. When viewed on a light table, the exposed autoradiographic signals reveal the location of ^{14}C -containing colonies on the overlaid master filter. The duplicate filter sheets were wrapped with cellophane and archived.

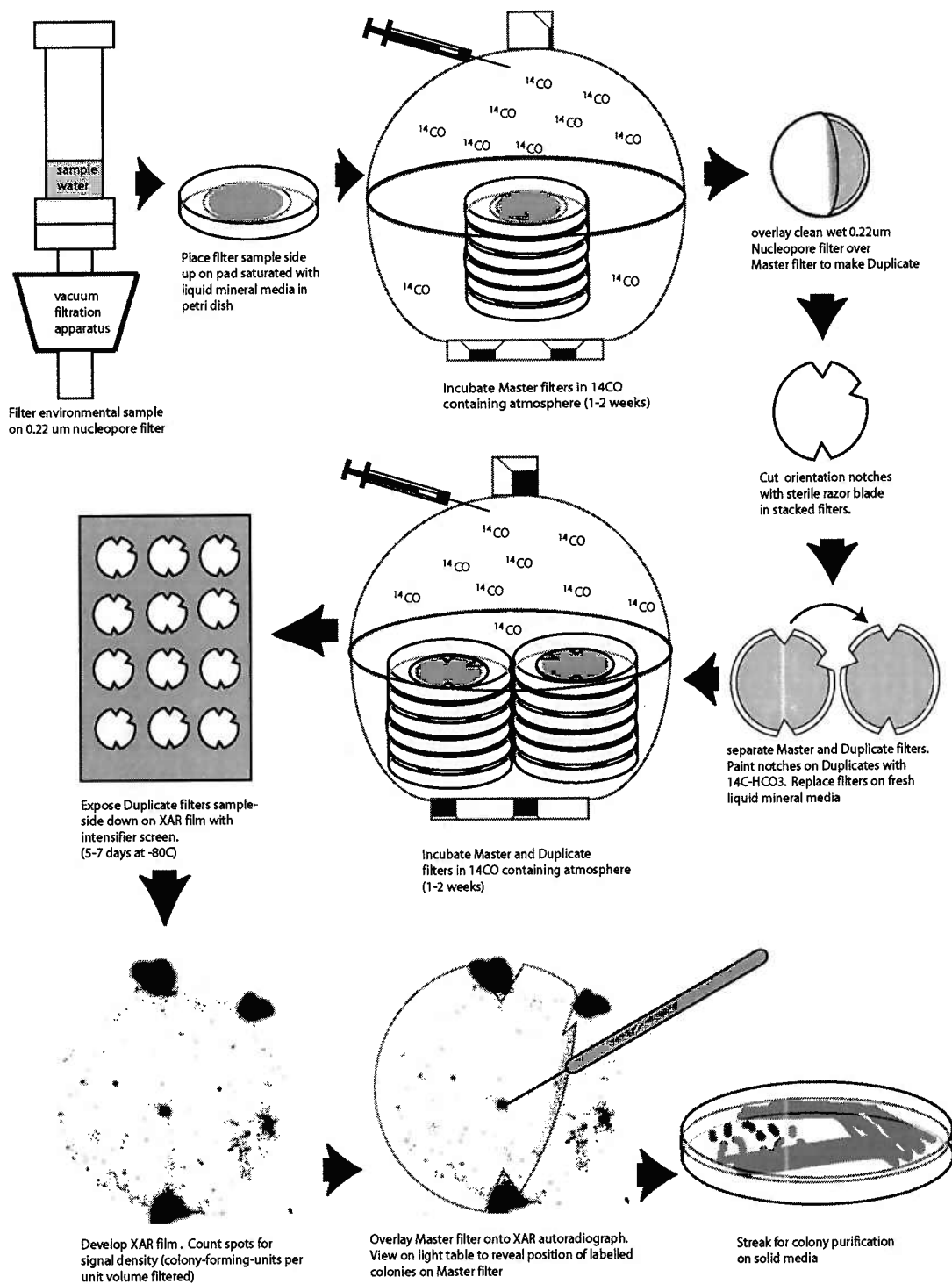
Exposed spots on the autoradiographic plates result from colonies that have incorporated carbon from ^{14}C -CO, and correspond to live colonies on the master filter. Selected colonies from the master filter were test-streaked on solid media (marine agar 2216) for purification, in anticipation that CO oxidizers are also heterotrophs. In this initial streaking, several colonies were obtained from each autoradiographically positive

colony sampled from the master membrane. Each of these were further isolated into pure cultures. Because at this point it was unknown which isolate conferred the positive signal in the initial autoradiography, each of these monocultures underwent another round of ^{14}C -CO incubation and autoradiography (Appendix B) to determine which pure-culture isolates incorporate labeled CO and conferred the original autoradiographic signal. Each of these isolates was given an alphanumeric designation and was noted for the media from which it was isolated in the initial incubation. Once positive CO-metabolizing isolates were determined, these were cross- tested for growth on alternate nitrogen-source media in a subsequent ^{14}C -CO incubation. This growth and screening protocol allowed the isolation of strains of organisms that incorporate label from CO when growing on carbon-rich media, and others that may incorporate label from CO when growing under oligotrophic conditions with either ammonium or nitrate as nitrogen source.

Specific CO oxidation rates of environmental isolates

Cell-specific CO-oxidation activity was determined with suspensions of selected strains at cell-densities measured by acridine orange direct counting (AODC) and incubated with ^{14}C -CO. Purified isolates were transferred from discrete colonies on 2216 agar and grown 1-2 days in 15 ml 2216 marine broth to a light turbidity ($\sim 10^7$ cells/ml). Liquid cultures were centrifuged for 10 min. at 10,000 RPM, and the broth was decanted leaving the pellet intact. The pellet was resuspended in 15 ml 0.22 μm filtered seawater to rinse, and centrifuged for 10 min. The rinse was repeated twice, and the pellet was

Figure 3.1: Autoradiographic procedure for isolating CO-metabolizing organisms from natural samples. Cells are deposited onto filters and incubated in a ^{14}C -CO atmosphere. After colony formation, duplicate filters are created and re-incubated with ^{14}C -CO. ^{14}C -containing colonies expose XAR film, and colonies are harvested from the master filters when oriented with the film.



finally re-suspended in 150 ml of aged and 0.22 μm filtered seawater ($\sim 10^6$ cell ml^{-1} final density). CO was injected into the headspace of each sealed bottle sufficient to maintain a dissolved [CO] of approximately 40 $n\text{M}$ in order to induce CO metabolic enzymes in each isolate. Cell density was determined after 2 days of CO induction and prior to the CO oxidation assay by direct microscopy (AODC). To measure the *in situ* rates of CO oxidation, to determine cell-specific rates of CO oxidation for selected isolates, and as a screening method to indicate ^{14}C -CO-oxidizing strains, we utilized the ^{14}C -CO oxidation method (Bartholomew & Alexander, 1979; Griffiths *et al.* 1982) described previously (Chapter 2). CO oxidation activity was calculated on a per-cell basis for each isolate ($\text{nmol CO oxidized cell}^{-1} \text{ hr}^{-1}$).

Molecular analysis.

CO metabolizing microorganisms isolated as pure cultures were picked and the 16S rRNA gene was amplified by PCR by a variety of treatments. In general, the universal bacterial primers 8f and 1492r were used in PCR amplification, resulting in a 1484 base-pair segment. The correct size of the product was confirmed by electrophoresis in 2% agarose gel run at 100 volts for 25-30 minutes, with either a Low DNA MASS Ladder or Amplisize Molecular Ruler 50-2,000 bp ladder. The gel was stained in ethidium bromide for 15-20 minutes, and viewed under UV and photographed using a ChemiImager low light imaging system (Alpha Innotech Corporation).

Most strains provided amplifiable template after picking solitary colonies from solid 2216 marine agar with a sterile toothpick and placed into 5 μl Lyse-N-Go PCR

reagent (Pierce), and a 2 min. heating period at 95°C prior to the addition of primers, nucleotides, and *Taq* polymerase. Other strains required a more stringent lysing procedure: 10 µl of liquid broth culture were centrifuged at 13,000 rpm for 4 min., and the supernatant removed. The pellet was washed once with MilliQ ultrafiltered water and resuspended into 5 µl Lyse-N-Go. The combination of osmotic shock and the Lyse-N-Go treatment of these more recalcitrant strains resulted in amplifiable template DNA. With one isolate (JT-10), rapid freeze-fracturing of cells in liquid nitrogen followed by treatment with CTAB and phenol/chloroform/isoamyl extraction (A. Teske, personal communication) was necessary to obtain a PCR product. In all amplification runs, a negative control containing no template and a positive control with 0.5 µl *E. coli* liquid culture were run to control for contamination and reagent quality. Amplified template was frozen or kept on ice. Template products were cleaned using the QIAquick PCR purification kit (Qiagen) protocol designed to purify fragments from 100 bp to 10 kb from primers, nucleotides, polymerases, and salts using spin columns in a microcentrifuge. Cleaned DNA was eluted with 50 µl H₂O.

In preparation for sequencing, the amount of PCR product needed was estimated by visual comparison of product band intensity (4 µl) to a low DNA mass ladder standard. Product volume required for sequencing reactions were adjusted to yield approximately 50 ng of DNA per reaction. The sequencing reactions were performed with the Big Dye Terminator (BDT) sequencing kit (Applied Biosystems) using 3 different bacterial primers in separate reactions. For each reaction, 1 µl BDT, 1 µl primer (8f, 1492r, or 519f), 50 ng DNA, and H₂O were combined to a total volume of 6 µl. 32

isolates and 3 separate primer reactions filled a 96-well microplate. The thermocycler was set for 25 cycles: 96°C for 10 sec., 50°C for 5 sec., and 60°C for 4 min.

The product was cleaned by isopropanol precipitation. The 96-well plate was removed from the thermocycler and spun briefly. Thirty μ l 75% IPA was added to each well, the plate was sealed well, and inverted to mix thoroughly. The plate was allowed to rest at room temperature for 15 min. The plate was centrifuged at 4170 RPM for 30 min. The sealant tape was removed and the plate was inverted carefully onto a paper towel to absorb IPA, taking care not to disturb the pellet. The pellet was washed by adding 50 μ l 70% IPA. The plate was resealed and spun at 3450 RPM for 10 min. The tape was removed and the plate inverted again to remove IPA. The inverted plate was centrifuged at 1950 RPM for 1 min. to bump down residual IPA. The plate was allowed to air dry for 20 min. Each pellet was resuspended in 7 μ l Hi-Di formamide. The plate was resealed with aluminum tape and stored at -20°C before sequencing with a ABI PRISM 3700 DNA Analyzer/Sequencer located at the Josephine Bay Paul Center, MBL. Sequences from 30 isolates were edited and assembled using Sequencher 4.0.5 (Gene Codes Corporation, Ann Arbor, MI). Sequencing resulted in double-stranded sequence fragments that were checked against the NCBI database using the BLAST search program (*Altschul et al.*, 1990) for initial identification.

RESULTS and DISCUSSION

Positive signals on XAR film were counted after two weeks of incubating in a ^{14}C -CO containing atmosphere at environmentally relevant levels of CO (40 - 50 ppm) (figure 3.4). Differential media plates were counted separately, and counts of colonies grown separately on nitrate and ammonium were combined to yield the cell density (cells ml^{-1}) that had incorporated ^{14}C from CO. The presence or absence of vitamins in the growth media made no significant difference in colony density counts (figure 3.4). Therefore, vitamin condition (“with vitamins” and “without vitamins”) values were averaged. Total plate counts were thus 395 ± 55 cells ml^{-1} in Vineyard Sound water (mean \pm SEM, $n=6$). Acridine orange direct counts (AODC) of Vineyard Sound water at the time of sampling were $2.71 \times 10^6 \pm 5.5 \times 10^4$ cells ml^{-1} (mean \pm SEM). Only a small fraction of cells present in the sample grew to visually distinct colonies under the experimental growth conditions (0.01% of total). Colonies that were able to grow under these conditions necessarily had respiratory adaptations that allowed them to grow in elevated [CO] conditions. It is possible that other cells having CO tolerance did not form colonies on the “solid” substrate (the membrane); therefore, our autoradiographic plate counts may underestimate CO metabolizing cells in coastal seawater due to this limitation.

To test the feasibility of this direct isolation approach to determine whether our acquired CO-metabolizing isolates are significant contributors to measured CO oxidation rate, we searched the primary literature sources to find specific activities of known CO-oxidizing strains. A range of published activities were selected for comparison with our

calculated CO-oxidation activities based on autoradiographic colony count. In an initial estimate of the proportion of total CO oxidation that can be attributed to organisms that produced colonies in a CO-containing atmosphere, our calculation used literature values for uptake efficiency (4% (Zavarzin & Nozhevnikova, 1977)), cellular carbon content (20 fg C cell⁻¹ (Cho & Azam, 1990)), and protein:dry weight conversion factor (1.4 mg protein per 3.1 mg dry weight (Cypionka *et al.*, 1980)). The amount of CO oxidized in a 25 ml liquid culture of these characterized strains at cell density of 1 x 10⁶ cells ml⁻¹ can be compared with CO oxidation rates we have observed in natural seawater samples (Table 3.2). Measurements of the CO oxidation rate of the coastal *in situ* assemblage are from 1-10 pmol CO hr⁻¹ 10⁻⁶ cells (total CO oxidation rate / total cell density by direct count), which is as active as the CO oxidation rate of one known carboxydotrophic organism, *Pseudomonas carboxydohydrogena* (Cypionka *et al.*, 1980). If 400 cells ml⁻¹ are actively metabolizing CO in the environmental samples, then the rate of CO oxidation by these cells increases to 2.5 – 25 nmol CO hr⁻¹ per 10⁶ cells, which is in the same order of magnitude as the most active described carboxydotrophic organisms.

Several strains carried over when colonies corresponding with the XAR film signal were picked from master filters and streaked on solid media for purification. To verify CO oxidizing activity for all membrane isolates, we reanalyzed purified cultures of each isolate by macroautoradiography, following the same method as the first round with few modifications concerning filter size and orientation. In autoradiographic exposures of purified isolates on different growth media (broth, low organic - NO₃, and low organic - NH₄), we expected to see most carbon assimilation from labeled CO in organisms that are

<u>strain</u>	<u>activity</u>	<u>CO oxidation rate</u> <u>(calculated)</u>	<u>reference</u>
<i>Pseudomonas carboxydoflava</i>	2300 $\mu\text{l CO hr}^{-1} \text{ mg protein}^{-1}$	18.12 $\text{nmol hr}^{-1} / 10^6 \text{ cells}$	<i>Cypionka et al.</i> 1980
<i>Comamonas compransoris</i>	2000 $\mu\text{l CO hr}^{-1} \text{ mg protein}^{-1}$	15.8 $\text{nmol hr}^{-1} / 10^6 \text{ cells}$	<i>Cypionka et al.</i> 1980
<i>Pseudomonas carboxydohydrogena</i>	100 $\mu\text{l CO hr}^{-1} \text{ mg protein}^{-1}$	0.788 $\text{pmol hr}^{-1} / 10^6 \text{ cells}$	<i>Cypionka et al.</i> 1980
Vineyard Sound assemblage		0.5– 10 $\text{pmol hr}^{-1} / 10^6 \text{ cells}$ (measured)	This study

Table 3.2: Literature values for specific CO oxidation rates of pure cultures of carboxydobacteria and of natural assemblages.

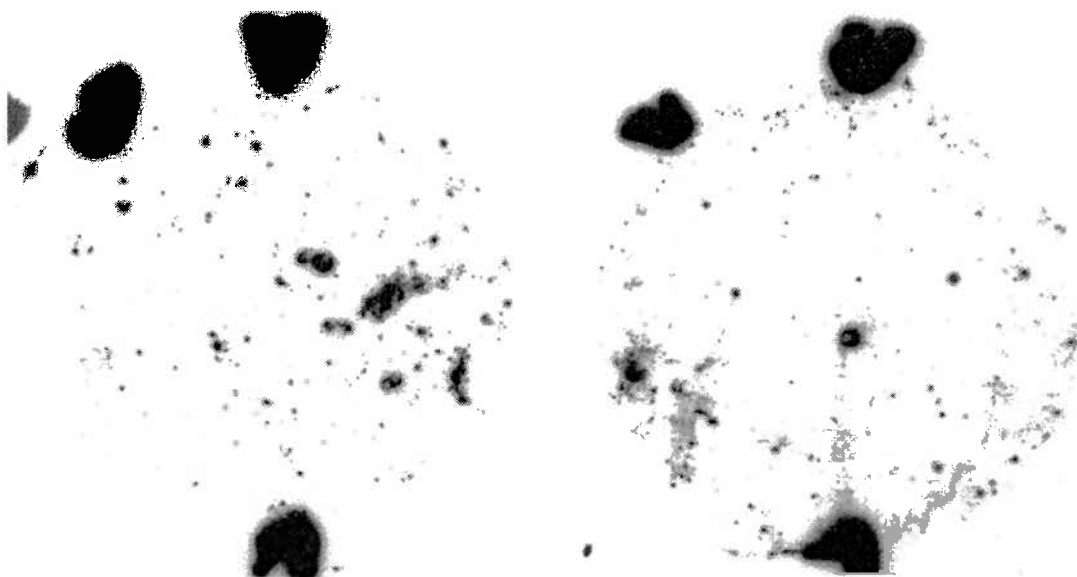


Figure 3.3: Representative autoradiographs of duplicate-filter holding colonies that incorporated labeled carbon from ^{14}C -CO. Volume filtered: 1ml, Vineyard Sound. Large painted notches are registration marks to allow alignment of master filters to film.

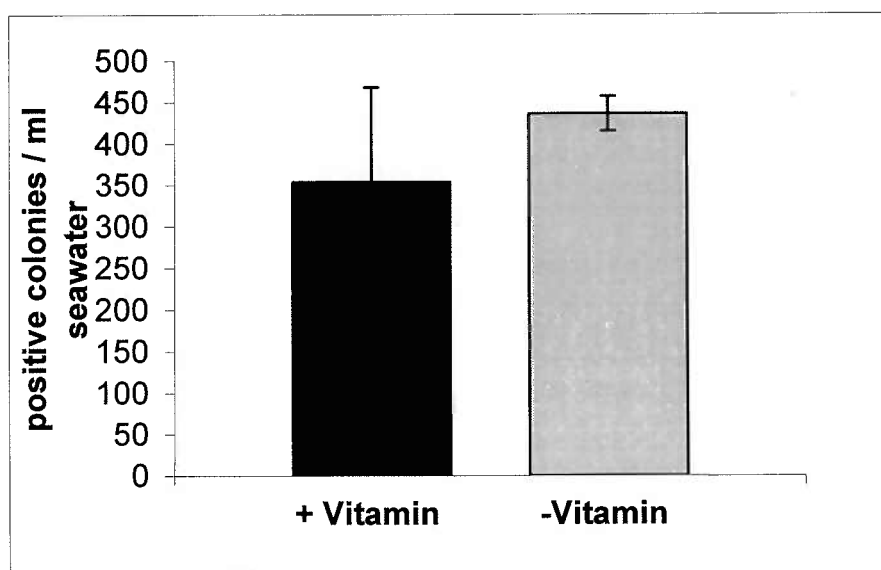


Figure 3.4: Uptake signals were counted separately on differential media. Nitrogen source values were combined, and ultimately the vitamin source values were averaged to yield 395 ± 55 positive colony-forming units per ml seawater.

<u>ISOLATE</u>	<u>BROTH</u>	<u>NO3</u>	<u>NH4</u>
JT-01	+	++	++
JT-02	+	+	+
JT-03	+		
JT-04	~	+	+
JT-05	+	~	+
JT-06a	++		
JT-06b	+		
JT-07	~		
JT-08	+		
JT-09	++	+	+
JT-10	+		
JT-11	+		~
JT-12	+	~	~
JT-13a	+	+	+
JT-13b	++		
JT-14	~	+	~
JT-15	+	+	
JT-16	++	+	
JT-17	+		
JT-18	+		
JT-19	++	~	~
JT-20	+		
JT-21	~		
JT-22	+		
JT-23	+		+
JT-24	+		+
JT-25	++		
JT-26	++	~	
JT-27	++	~	~
JT-28	++	~	~
JT-29	++	~	~
JT-30	+		
E. coli			

++ = entire filter black

+ = spot signals

~ = nearly invisible signal

Table 3.5: results of autoradiography of purified isolates incubated in a ^{14}C -CO-containing atmosphere (50 ppm) and different growth conditions (Appendix 2 for plates)

able to utilize CO in media that was poor in organic carbon, that is, heterotrophic organisms that induce CO-metabolic genes in response to an oligotrophic and CO-containing environment to supplement their carbon source. The surprising result of this second round of autoradiography with pure isolates was that every isolate strongly incorporated label from CO while growing under heterotrophic conditions (table 3.5). We searched the literature for precedents and possible explanations for this result.

In ^{14}C -CO₂ uptake experiments on the metabolically versatile heterotroph, *Sandaracinobacter sibiricus* [previously *Erythrobacter sibiricum* (renamed in Yurkov *et al.*, 1997)], by Yurkov and Beatty, 1998, a low level of CO₂ fixation was attributed to the enzyme phosphoenolpyruvate carboxylase. When intermediates of the citric acid cycle are removed during rapid biosynthesis, they are replaced by the anaplerotic fixation of CO₂ by this enzyme. If these organisms (our isolates) are oxidizing CO to CO₂ with CO-induced enzymes, then labeled CO₂ is readily available to fill these anaplerotic reactions, and therefore the cells produce a strong autoradiographic signal when grown in heterotrophic conditions.

Profitable oxidation of carbon monoxide during heterotrophic growth has also been reported in *Pseudomonas carboxydoflava*, a bacterium characterized as carboxydophilic when growing autotrophically in mineral medium in the presence of CO (Kiessling and Meyer, 1982). However, the authors report that virtually none of the carbon from CO was incorporated while growing in heterotrophic media; the oxidation of CO provided the organism with additional energy and stimulated growth rate and greater uptake of organic carbon compared to cultures grown in the absence of CO. Since *P.*

carboxydoflava profited from CO oxidation, 'cooxidation' is not suitable to describe this process. The term "mixotrophic" seems to be applicable to describe the profitable oxidation of CO by heterotrophically growing cells of *P. carboxydoflava*.

^{14}C -CO uptake experiments were conducted with pure isolates JT-01 and JT-08, the two most active CO-oxidizers resulting from our direct isolation program. The isolates were incubated in a ^{14}C -CO-containing atmosphere in liquid media for six days, with subsamples withdrawn periodically to determine the activity levels of ^{14}C that had been incorporated into cell matter and ^{14}C -CO oxidized into acid-volatile product. Two different liquid media were aged 0.22 μm -filtered seawater (collected 39°32'N, 68°W, 100m, E. Horgan) and 2216 marine broth (Difco), to compare the cells' label-uptake characteristics while growing under heterotrophic conditions versus carbon-limited conditions. Both CO-oxidizing isolates showed strong CO-oxidation activity when incubated either in seawater or in 2216 marine broth. When grown in seawater, ^{14}C -incorporation into particulate matter was undetectable over the entire six-day incubation period (figure 3.6, figure 3.7). When incubated in broth media, CO-oxidation reached a peak activity within 2 days (JT-01) or 3 days (JT-08) and label incorporation in particulate matter began to increase. When the relative fraction of total CO metabolized captured on filters was compared between the two isolates and their two different growth media, carbon from CO only become incorporated into particulate matter when the cells were in heterotrophic media. Cells grown in low-carbon media do not exhibit ^{14}C -accumulation from CO (figure 3.8), but oxidize CO rapidly. This is in

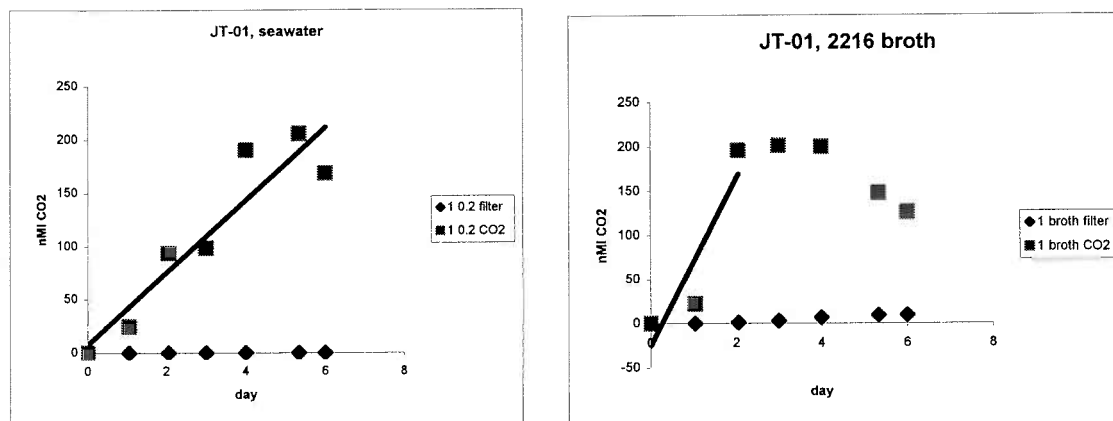


Figure 3.6: CO incorporated by isolate JT-01 over time while growing in carbon-limited media (left) or heterotrophic conditions (right). Filter activity (♦) and ¹⁴C-CO₂ (■) were measured separately at each time point.

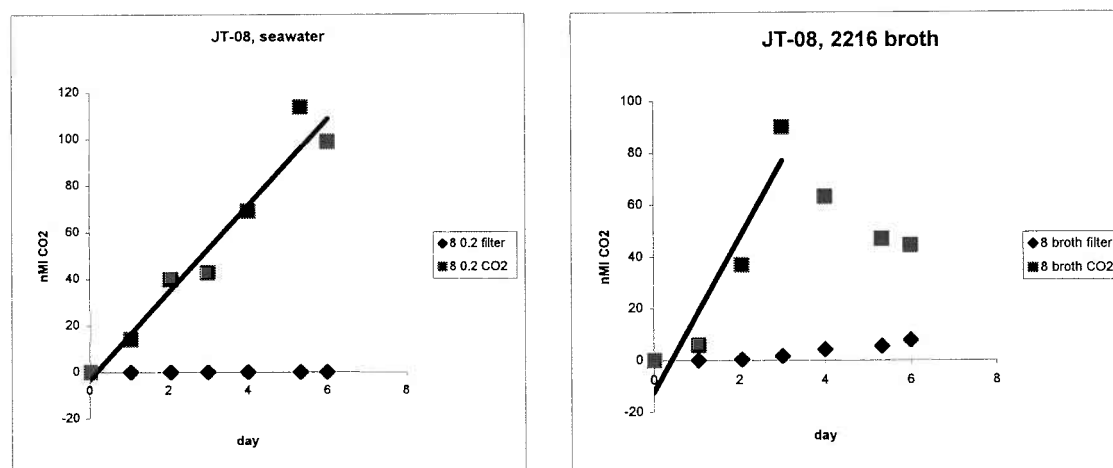


Figure 3.7: CO incorporated by isolate JT-08 over time while growing in carbon-limited media (left) or heterotrophic conditions (right). Filter activity (♦) and ¹⁴C-CO₂ (■) were measured separately at each time point.

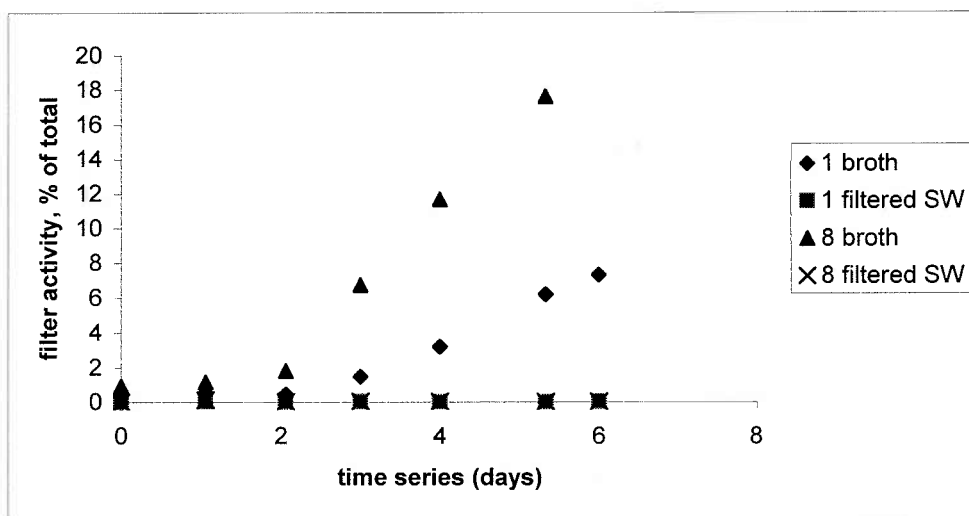


Figure 3.8: Fraction of total CO metabolism captured on filters for both JT-01 and JT-08, growing under two carbon conditions. JT-01 in filtered seawater (■), JT-01 in 2216 marine broth (◆), JT-08 in filtered seawater (×), and JT-08 in 2216 marine broth (▲).

agreement with the autoradiographic observations that isolates exhibit strong uptake of label when in broth as opposed to mineral media, and supports the hypothesis that carbon is entering the citric acid cycle as CO_2 in anaplerotic reactions to replace cycle intermediates that are removed during rapid biosynthesis.

Since the *in situ* microbial community did not incorporate label from CO when incubated in native seawater over the course of more than two days (figure 2.7), and two seawater isolates known to oxidize CO rapidly did not incorporate label from CO when grown in a carbon-poor seawater medium for up to six days (figure 3.8), we conclude that nearly all of the environmental CO that is acted upon by the microbial assemblage is oxidized to CO_2 and remains in solution as bicarbonate, and is not specifically incorporated into organic biomass other than indirectly by autotrophic organisms. Labeled bicarbonate need not be retained within the cells to confer a positive signal to XAR film, rather, it may be extracellular but still within the colony on the membrane.

To estimate the potential energetic benefit to mixotrophic cells oxidizing CO while utilizing environmental DOC, we searched primary literature sources for environmental concentrations of two bioavailable organic substrates, acetate and glucose, and calculated the energy advantage that mixotrophic organisms deriving energy from oxidation of CO at environmental levels (12 nM, this study) would have over organisms that lack this metabolic feature (Table 3.9). Free energy for each reaction was calculated as $\Delta G^0 = \sum \Delta G_f^0 (\text{products}) - \sum \Delta G_f^0 (\text{reactants})$. The calculated standard free energy yield was scaled down to the environmental concentration of the substrate. Energetic advantage was then calculated by dividing the free energy yield of CO oxidation at 12

Organic substrate	Environmental concentration (nM)	ΔG (kJ)	Energy advantage with CO oxidation
Acetate	20 (<i>Wu et al.</i> , 1994)	3.26×10^{-5}	19%
Glucose	8 (<i>Lee</i> , 1992)	2.3×10^{-5}	27%

Table 3.9: Energetic advantage of mixotrophs utilizing trace DOM as carbon sources and CO-oxidation as a supplemental energy source. Energy advantage is calculated by dividing the energy yield of CO oxidation at 12 nM [CO] by the energy yield of individual DOM species at their environmental concentrations (coastal).

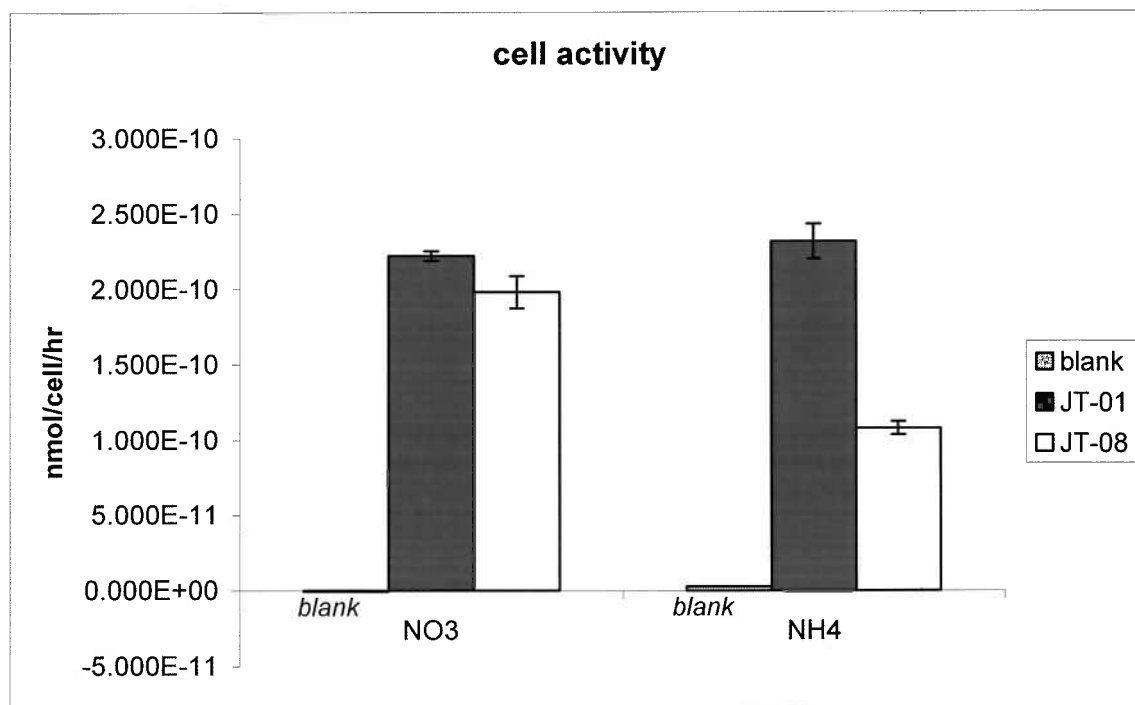


Figure 3.10: Cell-specific CO oxidation activity. The statistic used for comparing activities between isolates is specific activity in terms of nanomoles CO oxidized cell⁻¹ hour⁻¹, and is obtained by normalizing the CO oxidation rate by the cell density obtained by AODC. Error bars represent standard error of the mean. Isolates shown here are the most active CO oxidizers resulting from this study.

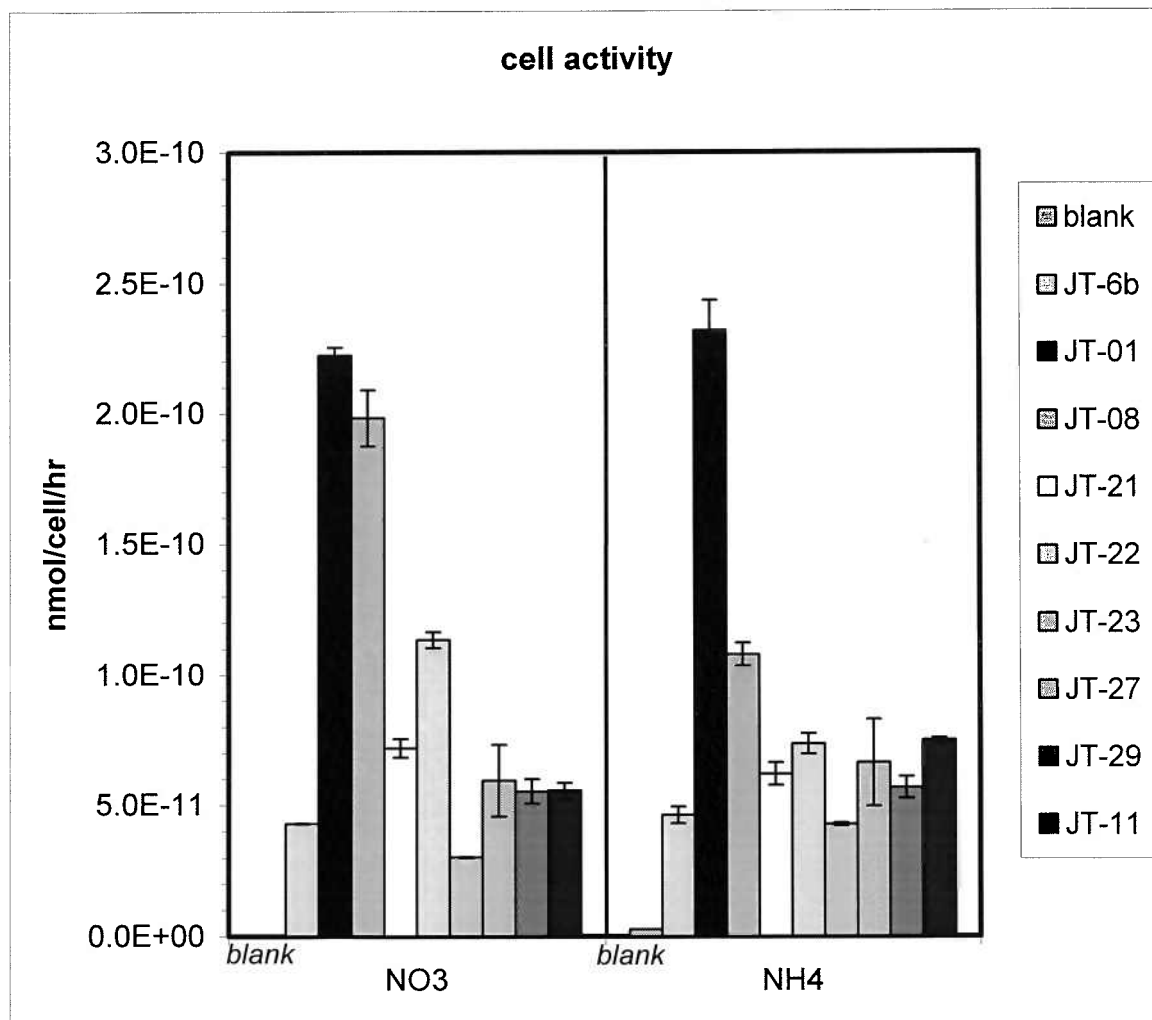


Figure 3.11 Environmental isolates tested for CO oxidation activity that have CO-oxidation greater than the average specific activity of the microbial assemblage in Vineyard Sound surface water (4.53×10^{-11} to 5.99×10^{-11} nmol CO oxidized cell⁻¹ hr⁻¹). Nine of 30 isolates oxidize carbon monoxide at significant rates. Error bars represent SEM. Close relatives based on BLAST returns of the isolate sequences are as follows: JT-6b, *Alteromonas* sp.; JT-01, *Ruegeria* sp.; JT-08, *Roseobacter* sp.; JT-21, *Cyclobacterium* sp.; JT-22, *Roseovarius* sp.; JT-23, γ -Proteobacter; JT-27, *Halomonas* sp.; JT-29, *Halomonas* sp.; JT-11, *Pseudomonas* sp.

nM by the free energy yield of the organic substrate oxidation. In each case, there is significant energy advantage to utilizing the energy from CO oxidation, even if CO-born carbon is not utilized and incorporated as biomass.

Isolate specific CO-oxidation rates

The specific rates of CO oxidation by the CO-oxidizing isolates recovered during this study were measured by the ^{14}C -CO oxidation assay performed with pure cultures. The direct measurement of the rate of CO oxidation (nM/hr) could not be used to compare between isolates because of differences in cell density between incubations. This raw statistic was normalized to cell density for each incubation (AODC) to yield a specific cell CO-oxidation activity that could be used to compare activities of separate isolates (nmol CO oxidized cell⁻¹ hr⁻¹) (figure 3.10). Specific CO oxidation activity was measured for every isolate in two different nitrogen-source conditions in mineral media (figure 3.11). In most cases, nitrogen source did not make a difference in whether organisms oxidized CO, or the magnitude of CO-oxidation activity if it existed. One isolate, (JT-08), oxidized CO at a higher rate while grown with nitrate as nitrogen source; this is the sole exception among 30 isolates and nine positive CO oxidizers. The overall mean CO oxidation activity for all positive isolates (n=9) was 8.953×10^{-11} nmol CO oxidized cell⁻¹ hr⁻¹. The average CO oxidation activity of the two most active isolates (JT-01 and JT-08) was more than 2-fold faster than the others (n=7) at 1.9×10^{-10} nmol CO oxidized cell⁻¹ hr⁻¹, and within range of the CO oxidation activity of one characterized carboxydotroph, *Pseudomonas carboxydohydrogena* (Cypionka et al., 1980).

Identity and Phylogeny.

After 16S-rDNA sequencing of 30 isolated strains, putative identifications based upon sequence and BLAST search returns were compiled (Table 3.12). It is important to note that while all isolates are CO tolerant to elevated concentrations of CO (40 - 50 nM), and are phylogenetically diverse, none of the 21 “carboxydotrophic” organisms described in literature are represented in our isolation program, which is consistent with the absence of any described carboxydotrophic organisms ever having been isolated from marine environments. Furthermore, all known carboxydotrophic organisms to date have been enriched under extremely high [CO] conditions (20% - 80%), where our direct isolation approach has used modestly high CO concentrations relative to those in the marine environment.

None of our 30 isolates are closely related to known ammonium-oxidizing organisms; nitrogen source did not affect CO oxidation rates in most cases except for JT-08, where CO-oxidation rate was reduced by half when the cells were in an NH_4^+ medium compared to those in a nitrate medium (Figure 3.11). The contribution of ammonium oxidizing bacteria (AOB) in CO oxidation in ocean water remains enigmatic. Prior work on these organisms to determine their role in environmental CO oxidation has been based mainly on comparisons of their K_M values in pure culture incubations with CO with kinetic values for CO consumption in ocean, soil, and lake samples. The ability of AOB to oxidize CO and their rates of CO consumption are negatively affected by the presence of ammonium, suggesting that CO and ammonium are competitive substrates and that CO is oxidized incidentally by ammonium monooxygenase. Furthermore, in the

ISOLATE	fragment	length (bp)	division	database match (BLAST)	% identity
1	8f-519r	(316)	<i>alphaproteobacteria</i>	<i>Paracoccus marcusii</i>	96%
	907f-1492r	(316)	<i>alphaproteobacteria</i>	<i>Paracoccus marcusii</i>	96%
2	8f-519r/519f-1492r	(689)	<i>gammaproteobacteria</i>	<i>Pseudomonas aeruginosa</i>	99%
3	8f-519r/519f-1492r	(800)	<i>gammaproteobacteria</i>	<i>Vibrio Harveyi</i>	99%
4	8f-519r/519f-1492r	(784)	<i>alphaproteobacteria</i>	<i>Marinobacter aquaeolei</i>	99%
5	8f-519r/519f-1492r		<i>gammaproteobacteria</i>	<i>Pseudomonas aeruginosa</i>	
6b	8f-519r/519f-1492r	(746)	<i>gammaproteobacteria</i>	<i>Glaciecola</i> sp.	95%
			<i>gammaproteobacteria</i>	<i>Pseudoalteromonas</i> sp.	95%
			<i>gammaproteobacteria</i>	<i>Alteromonas alvinellae</i>	95%
7	8f-519r/519f-1492r	(785)	<i>alphaproteobacteria</i>	<i>Ruegeria atlantica</i>	99%
			<i>alphaproteobacteria</i>	<i>Agrobacterium atlanticum</i>	98%
			<i>alphaproteobacteria</i>	<i>Agrobacterium meteori</i>	97%
8	8f-519r/519f-1492r	(729)	<i>alphaproteobacteria</i>	<i>Roseobacter</i> sp.	96%
			<i>alphaproteobacteria</i>	<i>Ruegeria atlantica</i>	96%
			<i>alphaproteobacteria</i>	<i>Agrobacterium atlanticum</i>	95%
			<i>alphaproteobacteria</i>	<i>Agrobacterium meteori</i>	95%
10	8f-519r/519f-1492r	(738)	<i>gammaproteobacteria</i>	<i>Pseudoalteromonas flavipulchra</i>	99%
			<i>gammaproteobacteria</i>	<i>Ps. piscicida</i>	99%
			<i>gammaproteobacteria</i>	<i>Ps. maricaloris</i>	99%
11	8f-519r/519f-1492r		<i>gammaproteobacteria</i>	<i>Pseudomonas aeruginosa</i>	99%
12	8f-519r/519f-1492r	(687)	<i>gammaproteobacteria</i>	<i>Pseudomonas aeruginosa</i>	99%
13a	8f-519r/519f-1492r	(1398)	<i>gammaproteobacteria</i>	<i>Pseudomonas aeruginosa</i>	98%
13b	8f-519r/519f-1492r	(732)	<i>epsilonproteobacteria</i>	<i>Micrococcus luteus</i>	99%
			<i>epsilonproteobacteria</i>	<i>Arthrobacter</i> sp.	99%
			<i>epsilonproteobacteria</i>	<i>Micrococcus psychrophilum</i>	98%
14	8f-519r/519f-1492r	(821)	<i>alphaproteobacteria</i>	<i>Ruegeria atlantica</i>	99%
			<i>alphaproteobacteria</i>	<i>Agrobacterium atlanticum</i>	98%
			<i>alphaproteobacteria</i>	<i>Agrobacterium meteori</i>	98%
15	8f-519r/519f-1492r	(740)	<i>alphaproteobacteria</i>	<i>Silicibacter pomeroyi</i>	95%
16	8f-519r/519f-1492r	(782)	<i>Flavobacteria</i>	Uncultured <i>Cytophagales</i>	95%
			<i>Flavobacteria</i>	<i>Cytophaga</i> sp.	95%
17/18	8f-519r/519f-1492r	(683)	<i>Flavobacteria</i>	<i>Oceanomonas baumannii</i>	98%
19	8f-519r/519f-1492r	(584)	<i>gammaproteobacteria</i>	<i>Halomonas marina</i>	100%
20	8f-519r/519f-1492r	(779)	<i>alphaproteobacteria</i>	<i>Ruegeria atlantica</i>	99%
			<i>alphaproteobacteria</i>	<i>Agrobacterium atlanticum</i>	98%
			<i>alphaproteobacteria</i>	<i>Agrobacterium meteori</i>	98%
21	8f-519r/519f-1492r	(279)	<i>Flavobacteria</i>	<i>Cyclobacterium marinum</i>	99%
22	8f-519r	(741)	<i>alphaproteobacteria</i>	<i>Roseobacter</i> sp.	97%
23	8f-519r/519f-1492r	(270)	<i>gammaproteobacteria</i>	Uncultured <i>Neptunomonas</i>	91%
			<i>gammaproteobacteria</i>	<i>Thiomicrospira</i> sp.	91%
			<i>gammaproteobacteria</i>	<i>Methylomonas methanica</i>	91%
25	8f-519r/519f-1492r	(804)	<i>Flavobacteria</i>	CFB group bacterium	100%
26	8f-519r/519f-1492r	(725)	<i>Methanobacterium</i>	<i>Zobellia uliginosum</i>	96%
27	8f-519r	(592)	<i>gammaproteobacteria</i>	<i>Halomonas alimentaria</i>	96%
		(552)	<i>gammaproteobacteria</i>	<i>Halomonas salina</i>	97%
28	8f-519r/519f-1492r	(686)	<i>gammaproteobacteria</i>	<i>Halomonas marina</i>	100%
29	8f-519r/519f-1492r	(679)	<i>gammaproteobacteria</i>	<i>Halomonas marina</i>	100%
30	8f-519r/519f-1492r	(316)	<i>Flavobacteria</i>	Uncultured <i>Cytophagales</i>	97%

Table 3.12: BLAST Identification of Isolates. **Bold text** denotes CO-oxidizing isolates.

Type strain tested	Source collection	Accession number
<i>Paracoccus versatus</i>	DSMZ	582
<i>Ruegeria atlantica</i> = <i>Agrobacterium atlanticum</i>	DSMZ	5823
<i>Ruegeria gelatinovorans</i>	DSMZ	5887
<i>Paracoccus denitrificans</i>	DSMZ	7001
<i>Roseobacter litoralis</i>	DSMZ	6996
<i>Paracoccus alcaliphilus</i>	DSMZ	8512
<i>Paracoccus aminophilus</i>	DSMZ	8538
<i>Ruegeria algicola</i>	DSMZ	10253
<i>Paracoccus pantotrophus</i>	DSMZ	11072
<i>Silicibacter lacuscaerulensis</i>	DSMZ	11314
<i>Sulfitobacter brevis</i>	DSMZ	11443
<i>Roseovarius tolerans</i>	DSMZ	11457
<i>Sulfitobacter mediterraneus</i>	DSMZ	12244
<i>Roseobacter gallaeciensis</i>	DSMZ	12440
<i>Pseudomonas aeruginosa</i>	ATCC	10145
LFR (<i>Ledyard et al.</i> , 1993)	ATCC	51258
EE-36	M.A.Moran (UGA)	
<i>Silicibacter lacuscaerulensis</i>	M.A.Moran (UGA)	
<i>Silicibacter pomeroyi</i> DSS-3	M.A.Moran (UGA)	
ISM	M.A.Moran (UGA)	
NF18	Jannasch collection (WHOI)	
TB66	Jannasch collection (WHOI)	
AIII3	A. Teske (WHOI)	
DI4	A. Teske (WHOI)	
DIII4	A. Teske (WHOI)	

Table 3.13: Type cultures tested for CO-oxidation activity, their source, and accession numbers where applicable.

experiments performed by Jones and Morita (1983) with *Nitrosococcus oceanus* and *Nitrosomonas europaea*, they were unable to obtain K_M values as low as those reported for ocean, soil, or lake samples (4 – 9 nM)(Conrad and Seiler, 1982), even after 24 hours of nutrient starvation (222 nM for *N. europaea*, 889 nM for *N. oceanus*). It is not known what advantage the ammonium oxidizers get from their cooxidation of CO, if any, since they do not incorporate CO-born carbon and the energy of CO oxidation by AMO is not coupled to the electron transport system.

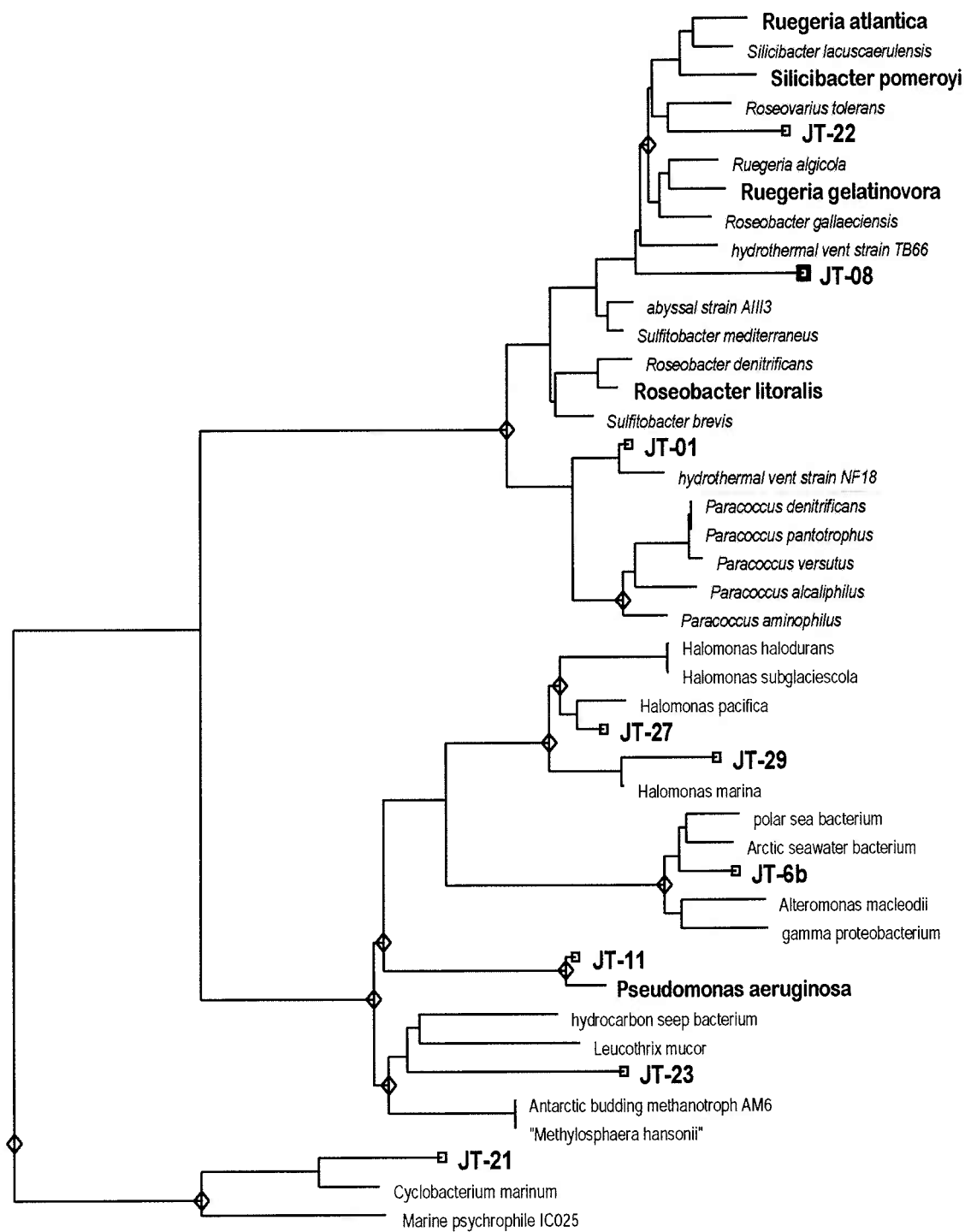
Phylogenetic analysis based on the 16S-rDNA sequences of positive CO-oxidizing isolates places the most active strains (JT-01, JT-08, JT-22) in the *Roseobacter* group (González *et al.*, 1999) and *Paracoccus* group of the α -subclass of the *Proteobacteria* (Figure 3.14). We obtained and tested type strains belonging to the *Roseobacter* and *Paracoccus* groups (Table 3.13) for CO-oxidation activity using the ^{14}C -CO oxidation assay, and discovered that four *Roseobacter* type organisms oxidized CO at environmentally relevant levels: *Roseobacter litoralis*, *Ruegeria atlantic*, *Ruegeria gelatinovora*, and *Silicibacter pomeroyi* (Figure 3.14).

Organisms belonging to the *Roseobacter* group (including the genera *Rhodovulum*, *Roseivivax*, *Roseobacter*, *Roseovarius*, *Rubrimonas*, *Ruegeria*, *Sagittula*, *Stayelam* *Stappia* *Silicibacter*, and *Sulfitobacter*) have been reported by Gonzalez and Moran (1997) as contributing up to 28% of the total 16S ribosomal DNA (rDNA) sequences in seawater samples from the coast of the southeastern United States. *Roseobacter* species have been reported as being a dominant microbial component during a North Sea coccolithophore bloom, contributing 24% of the bacterioplankton numbers

and up to 50% of the biomass (Zubkov *et al.*, 2001). *Roseobacter* has also been determined to contribute greater than 30% of total SSU rDNA in the surface waters of the Monterey Bay upwelling plume (Suzuki *et al.*, 2001). Given the rate of CO-oxidation by some *Roseobacter* group isolates and their probable numerical density in the study location, *Roseobacter* group organisms are likely important contributors to total CO oxidation in coastal waters. Many members of the *Roseobacter* group acquired from various sources (Table 3.13) tested for CO-oxidation activity were negative for that phenotype (Figure 3.15); CO oxidation is therefore not a characteristic phenotype of the marine alpha, *Roseobacter*, or *Paracoccus* clades. However, the most active of the organisms isolated in this study are representatives of *Roseobacter* and *Paracoccus*. While our CO-oxidizing isolates are phylogenetically very diverse, they all are aerobic heterotrophs and halotolerant. Descriptions of the of genera to which the CO oxidizing isolates resulting from this study belong are excerpted from Bergey's Manual of Determinative Bacteriology, 9th ed. and may be found in Appendix C.

To estimate the fraction of total CO-oxidation activity measured in natural seawater that can be attributed to organisms of the *Roseobacter* group, the specific activities of *Roseobacter* group organisms obtained from our isolation program and the CO-oxidizing *Roseobacter* type cultures were averaged. The average specific activity of the seven *Roseobacter* CO oxidizing strains (JT-01, JT-08, JT-22, *Roseobacter litoralis*, *Ruegeria gelatinovora*, *Silicibacter pomeroyi*, *Ruegeria atlantica*) was 1.07×10^{-10} nmol CO cell⁻¹ hr⁻¹. The average cell density of the Vineyard Sound coastal sampling location is 1.96×10^6 cells ml⁻¹. As *Roseobacter* group organisms have been found to compose

Figure 3.14: Neighbor-joining tree including positive CO-oxidizing isolates from this study (JT-*) in their phylogenetic context. **Bold** type denotes strains that have tested positive for CO oxidation. *Italicized* type denotes type cultures that have been tested and found negative for CO oxidation.



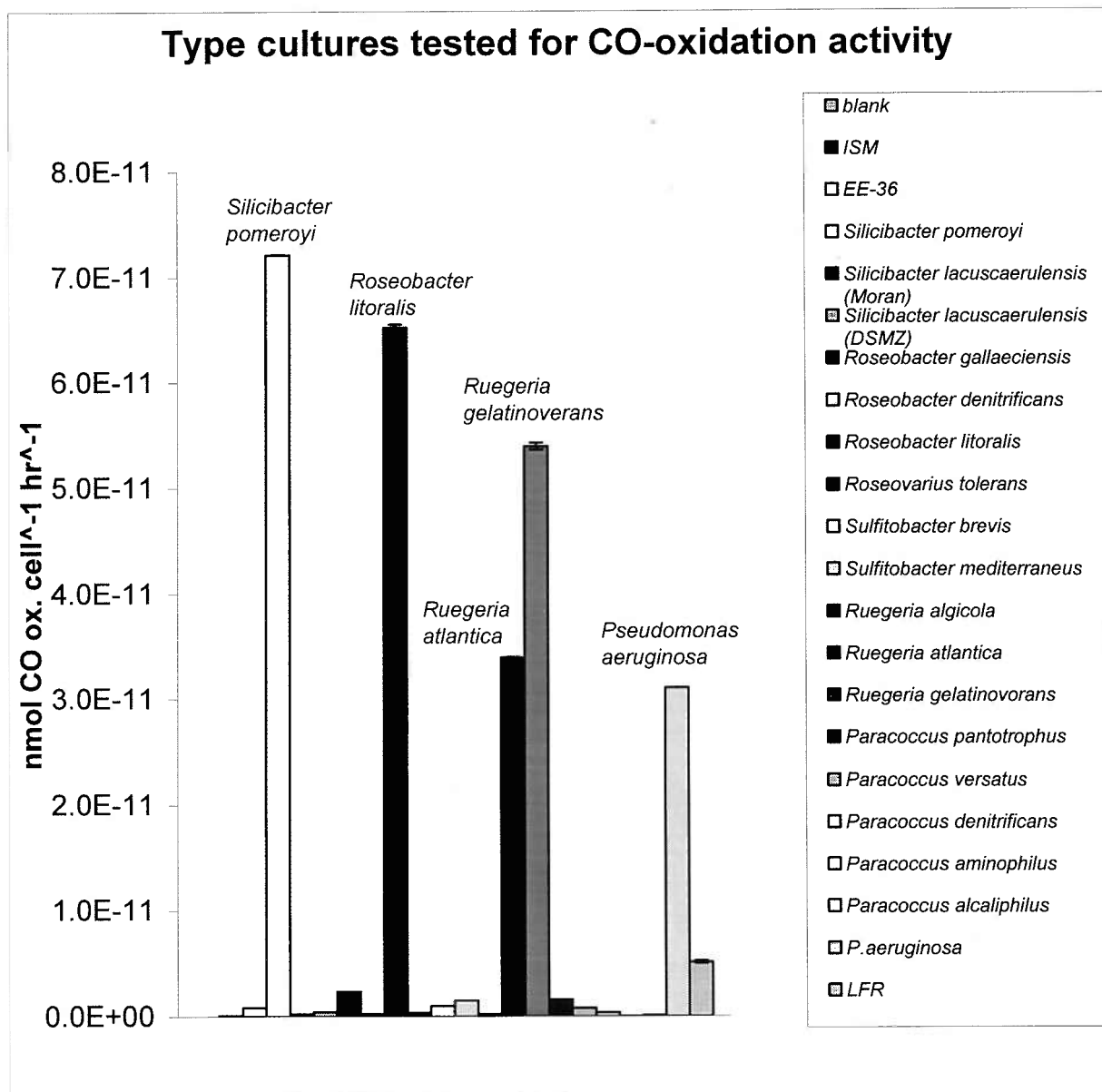


Figure 3.15: Type culture CO-oxidation specific activities. Four of 12 type species tested in the *Roseobacter* group and one strain of *P. aeruginosa* oxidize carbon monoxide with environmentally relevant specific activities.

28% of a natural coastal microbial population (Gonzalez and Moran, 1997) and greater in other environments, this percentage is a conservative value for the relative abundance of *Roseobacter* at our sampling site. The rate of CO oxidation that would result from this average specific activity at this relative cell density would be 0.06 nM CO oxidized hr⁻¹. This rate value, once normalized to 1 nM incubation [CO] yields a rate constant $k = 0.033 \text{ hr}^{-1}$. The average rate constant measured in bulk water samples from Vineyard Sound in $0.064 \pm 0.021 \text{ hr}^{-1}$. Therefore, members of the marine alpha subgroup of the *Proteobacteria* may potentially contribute 50% of the total CO oxidation that was measured in natural seawater samples. The sole parameter in this calculation not directly measured was the relative abundance of marine-*alpha* group cells at our sampling location; to further constrain the contribution of *Roseobacter* (or other specific groups) to environmental CO oxidation, it was necessary to enumerate these cells in the local environment and determine their relative proportion to the total assemblage, and to determine the relative proportion of *Roseobacter* that are CO-metabolically active. This topic is addressed in Chapter 4 and will consist of a triple-labeling technique (Substrate-Tracking AutoRadiography / Fluorescent *In Situ* Hybridization, or STARFISH) that will allow the simultaneous quantification and discrimination of total cell density, CO-metabolizing cells, and marine alpha-type cells in the same microscopic field.

The Class *alphaproteobacteria* currently contains several organisms that have been previously identified as “carboxydophilic”, though many were originally members of different taxonomic classes because of their metabolic features and have since been reclassified based on their molecular phylogeny. An example is the reclassification and

renaming of *Pseudomonas carboxydovorans*, formerly in the *gamma* subdivision of *Proteobacteria*, to *Oligotropha carboxidovorans*, currently in the *alpha* subdivision (Meyer *et al.*, 1993b). *O. carboxidovorans* has become a model species for studies of the CODH enzyme and *cox* genes that confer CO metabolism. *Carbophilus* and *Zavarzinia*, are two other examples of genera that are carboxydophilic and currently classified within *alphaproteobacteria* that were formerly misclassified as *Alcaligenes* (*betaproteobacteria*) and *Pseudomonas* (*gammaproteobacteria*), respectively.

The key enzyme in the aerobic utilization of CO by many described carboxydophilic microorganisms is carbon monoxide dehydrogenase (CODH), which catalyzes the oxidation of CO to CO₂ (Meyer *et al.*, 1982, 1993a). The CODH structural genes *coxMSL* of *Oligotropha carboxidovorans*, an *alphaproteobacteria* (formerly *Pseudomonas carboxydovorans* [Meyer *et al.*, 1993b]), have been sequenced (Schübel *et al.*, 1995) and are part of a *cox* gene cluster *coxBCMSLDEFGHIK* (Santiago *et al.*, 1999), which resides on the 128-kb megaplasmid pHCG3. The crystal structure of CODH has been solved at a 2.2 Å resolution (Dobbek *et al.*, 1999). CODH is composed of an 88.7-kDa molybdopterin (L), a 30.2-kDa flavoprotein (M) (Gremer *et al.*, 2000), and a 17.8-kDa iron-sulfur protein (S) in a (LMS)₂ subunit structure. The *cox* genes are specifically and coordinately transcribed under chemolithoautotrophic conditions in the presence of CO as carbon and energy source. All deduced products of the *cox* genes of *O. carboxidovorans* have counterparts in so far uncharacterized gene clusters of *Pseudomonas thermocarboxydovorans*, *Hydrogenophaga pseudoflava*, *Bradyrhizobium japonicum*, and *Mycobacterium tuberculosis*. In addition to the clustered *cox* genes,

mutational analyses have identified the genes *lon*, *cycH* and *orfX* which reside on the plasmid pHCG3. The Lon protease, the CycH protein and the unknown *orf* gene product have essential functions in the utilization of CO (Santiago *et al.*, 1999).

The similarity among the CODH from otherwise phenotypically diverse carboxydobacteria is striking (Meyer & Schlegel, 1983; Kim *et al.*, 1982) and suggests a common evolutionary origin for the CODH genes and relatively recent dispersal of the trait, perhaps by plasmid-mediated genetic exchange. Plasmids are readily mobilizable between taxa and represent the most common method of acquiring selectable traits (e.g. antibiotic resistance determinants). Plasmids are rarely integrated into the chromosome, therefore the acquired trait(s) must confer an advantage sufficient to overcome not only inactivation by mutation, but also elimination by segregation. There are, however, several proposed mechanisms for DNA assimilation into the bacterial genome, including *a)* persisting as a episome; *b)* homologous recombination, which is likely to serve primarily to re-assort variation among closely related taxa and is unlikely to allow introduction of novel traits; *c)* integration mediated by bacteriophage integrases or mobile element transposases; and *d)* illegitimate incorporation through chance double-strand break repair (Ochman *et al.*, 2000).

In a genome sequencing project for the *Roseobacter* isolate *Silicibacter pomeroyi* strain DSS-3, preliminary sequence data have shown the *cox* genes *coxS*, *coxL*, and *coxM*, plus many of the accessory *cox* proteins (D, E, F, G), arranged in an operon (M.M. Moran, personal communication). The genes have greatest similarity to those of *O. carboxidovorans*, but also high similarity to *H. pseudoflava* and *B. fungorum* genes.

Silicibacter possesses a 500-kb megaplasmid similar to *Oligotropha* (along with a number of other *alphaproteobacteria*), however, the *cox* gene cluster is not located there. The phenotype may be transmissible to other species through lateral gene transfer even if the operon is not carried on a plasmid, but rather in a transposable element within the chromosome flanked by two insertion sequences. It may be mobilized as a complex transposon that can integrate into the chromosomes of phylogenetically diverse bacterial species (Kleckner, 1989). This mode of lateral gene transfer is consistent with the appearance of the CO oxidation phenotype in widely diverse organisms but not as a characteristic shared within or between closely related taxa, and the presence of the *cox* gene cluster in the genome rather than in a plasmid in *Silicibacter pomeroyi*.

Lateral gene transfer involves the introduction of DNA into a single lineage, and the acquired trait is limited to the descendants of the recipient strain and absent from closely related taxa, thereby producing a scattered phylogenetic distribution. Lateral gene transfer need not be invoked to explain the sporadic occurrence of certain phenotypic traits, such as antibiotic resistance, because these properties can originate through point mutations in existing genes and therefore may evolve independently in divergent lineages (Ochman *et al.*, 2000). Thus, additional information is needed to discriminate between convergent evolution and lateral gene transfer. Clearly, the strongest evidence for (or against) lateral gene transfer of the *cox* operon derives from a molecular genetic analysis of the *cox* gene sequences themselves.

Primer sequences specific for *cox* genes required for active CODH and the ability to oxidize CO may be designed and used to amplify and confirm the presence of these

genes in marine isolates known to oxidize environmental CO. If the CO oxidation phenotype correlates with the presence of *cox* genes and their products, then the biochemical mechanism of CO oxidation in marine environments will be better understood. Phylogentic distribution of the phenotype may also be explained by sequence analysis of the *cox* genes in CO oxidizing isolates or cloned from environmental samples. Furthermore, molecular probes specific for conserved *cox* genes may enable a direct quantification of diverse CO-oxidizing cells in marine environments.

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Chapter IV:

Group-specific enumeration of CO-oxidizing microorganisms in natural samples by Substrate Tracking Microautoradiography - Fluorescent In Situ Hybridization (STARFISH)

ABSTRACT

Fluorescent in situ hybridization (FISH) using oligonucleotide probes specific for the *Roseobacter* and other subclasses of the α -Proteobacteria (the marine alpha group) was used in combination with microautoradiography to detect and enumerate microorganisms from natural seawater sources that are capable of metabolizing carbon from labeled carbon monoxide (CO) and are members of a specified phylogenetic group. Marine alpha group organisms were a major component of total cell numbers (45.7%) at the time of sampling (March 2003), and CO-oxidizing members of the marine alpha group contribute up to 40.7% of total CO oxidation in coastal waters.

INTRODUCTION

A challenge in microbial ecology is to link the metabolic activity of microorganisms in natural waters under conditions approaching those *in situ* with their phylogenetic identity. A composite microbial population may be studied by nonselective methods that measure gross metabolism, or by selective measurements such as the uptake of radiolabeled substrates or the growth of culturable bacteria. Quantifying the activity of microbial populations as a whole, however, yields no information on the structure of the population in terms of the number of microorganisms and the specific fraction of the total

number engaged in the measured function. Specific activity indices, introduced by Wright (1978), express metabolic activity parameters per organism by dividing the activity term (^{14}C -CO oxidation rate, in our study) measured for the composite microbial population by total microscopic direct counts (DC) for samples. This approach represents a first approximation for the interpretation of the relationship between measured microbial activities and a structural component of the population, i.e., the number of microorganisms (Tabor & Neihof, 1982).

Microautoradiography has the potential to address a number of specific questions relating to substrate uptake by natural populations of bacteria and has been applied to the study of bacterial activity in a number of distinct environments (Grey *et al.*, 1999). The use of radiolabeled substrates in combination with microautoradiography does allow analysis of the metabolic activity of prokaryotes under conditions that approach *in situ* conditions by direct visualization of microorganisms with active substrate uptake systems within a complex community. A major limitation of microautoradiography has been an inability to correlate the activity detected with identification and detection of a responsible organism. One attempt to solve this problem has involved combining microautoradiography with fluorescent *in situ* hybridization.

In situ characterization of the marine picoplankton “black box” has been restricted mostly to determinations of community composition by a number of molecular techniques, including fluorescent *in situ* hybridization (FISH) (Amann *et al.*, 1990, 1995; DeLong & Shah, 1990, DeLong *et al.*, 1999, Gonzolez & Moran, 1997). In FISH fluorescently labeled oligonucleotide probes are used to identify and enumerate

microorganisms *in situ* (DeLong *et al.*, 1989). Probe sequences can target organisms at various taxonomic levels; some probes are universal and target all organisms (Giovannoni *et al.*, 1988), while others are for the domains *Archaea* (Massana *et al.*, 1997) and *Bacteria* (Giovannoni *et al.*, 1988). Other probes target intermediate levels, for example, the α subdivision of the class *Proteobacteria* (Manz *et al.*, 1992; Ouverney & Fuhrman, 1999).

The objective of this study was to utilize a method that involves a direct combination of FISH performed with rRNA gene-targeted oligonucleotide probes and microautoradiography for simultaneous *in situ* identification and determination of substrate uptake patterns of individual microbial cells within a natural microbial assemblage (Lee *et al.*, 1999). Here FISH and microautoradiography are combined on a single slide to determine the proportion of the total microbial assemblage that has incorporated carbon from CO in specific picoplankton groups. The cells are triple-labeled with a ^{14}C -labeled substrate, a group-specific oligonucleotide probe, and a general DNA stain. Within the same microscopic field it is possible to determine the percentage of a specific prokaryotic phylogenetic group in a mixed sample and the distribution of substrate uptake within the target subgroup (Ouverney & Fuhrman, 1999).

In our program to isolate and cultivate CO metabolizing organisms in a coastal marine environment (Chapter 3), we discovered many of the active CO oxidizers belonged to the *Roseobacter* clade. The '*Roseobacter* group' as recently referred to by González *et al.* (1999), encompasses a physiologically and geographically diverse group of bacteria within the α -subclass of the *Proteobacteria* (Brinkmeyer *et al.*, 2000), and

together with *Paracoccus* composes the “marine alpha group”. These heterotrophic bacteria are strongly implicated in the turnover of the climatically active gas, dimethylsulphide (DMS), and particularly its precursor, an algal osmolyte dimethylsulphonopropionate (DMSP) (Ledyard *et al.*, 1993; González *et al.*, 1999). *Roseobacter* group organisms were found to be active in lignin transformation (González *et al.*, 1997) and methyl and inorganic sulfur compound utilization (Sorokin *et al.*, 1995; González *et al.*, 1997). Bacteria of this clade have been discovered in a wide range of marine habitats including pelagic environments (Eilers *et al.*, 2000; Zubkov *et al.*, 2001; Bano & Hollibaugh, 2002), coastal seawater (González & Moran, 1997; Eilers *et al.*, 2001; Pernthaler *et al.*, 2002; Suzuki *et al.*, 1997; this study), sediments (Sobecky *et al.*, 1998), marine snow aggregates (Rath *et al.*, 1998; Gram *et al.*, 2002) and as endosymbionts or in association with microalgae (Ashen & Goff, 1996, 2000; Shiba, 1991) and invertebrates (Barbieri *et al.*, 1996; Ruiz-Ponte *et al.*, 1998). *Octadecabacter antarcticus* and *O. arcticus* (Gosink *et al.*, 1997) isolated from polar sea ice, *Antarctobacter heliothermus* (Labrenz *et al.*, 1998), and *Roseovarius tolerans* (Labrenz *et al.*, 1999) isolated from a hypersaline lake in Antarctica, and *Silicibacter lacuscaerulensis* (Petursdottir & Kristjansson, 1997) isolated from a geothermal lake in Iceland represent members of this clade found in extreme environments. *Roseobacter* group bacteria have also been isolated from cultures of toxic dinoflagellates (Lafay *et al.*, 1995). Furthermore, the SAR83 (Giovannoni *et al.*, 1990) cluster of organisms detected in the oligotrophic gyre of the Sargasso Sea have very similar 16S-rDNA sequences to known *Roseobacter* organisms that are readily cultured in the laboratory (Amann, 2000).

This seemingly ubiquitous group of microorganisms may play an important role in the bio-oxidation of photolytically produced CO in various marine environments, and their potential CO metabolic activity has previously been overlooked in other characterization studies.

METHODS and MATERIALS

Fluorescent In Situ Hybridization and stringency testing.

Liquid suspensions of type species *Paracoccus denitrificans* (DSMZ #6610) and type strain *Roseobacter litoralis* (DSMZ #6996) in 2216 marine broth grown for 24 hours at 20°C were used for FISH stringency testing. The cultures were centrifuged at 9000 rpm for 5 minutes, and the pellets were resuspended in 0.22 µm-filtered seawater to rinse. The suspension was again centrifuged at 9000 rpm for 5 min., and the pellets were resuspended in 250 µl PBS and 750 µl 4% paraformaldehyde solution. FISH was performed under various hybridization conditions to empirically determine the formamide concentration of the hybridization buffer that would allow discrimination between *Roseobacter* target cells and *Paracoccus* cells, having a single and consistent mismatch in the probe sequence. The *Roseobacter* clade-specific oligonucleotide probe used for hybridization was ROSEO536R (*Brinkmeyer et al.*, 2000). The probe sequence (5' – 3') CAACGCTAACCCCCTCCG was constructed with a 5'-Cy3 label and HPLC-purified by Thermo Hybaid (former Interactiva, Ulm, Germany). At high formamide concentrations (40%) in the hybridization buffer (HB), we were able to discriminate between *Roseobacter* target cells and *Paracoccus* cells having the single mismatch to the

labeled probe. 40% formamide was used in FISH HB in combination with the competitor probe ROSEOC536R ((5' – 3') CAACGCTAGCCCCCTCCG) to further suppress false positive signals. The ROSEOC536R probe specificity was checked against the RDP II database (<http://rdp.cme.msu.edu/html/analyses.html>) using the “Probe Match” function, and was found highly specific for the *Roseobacter* group. Type discrimination was confirmed via FISH and a mixed binary culture of *Roseobacter litoralis* and *Paracoccus denitrificans*, and the ROSEOC536R/ROSEOC536R combination.

In enumerating *Paracoccus*, the ROSEOC536R sequence was Cy3-labeled and designated “PARA536R”, and the ROSEOC536R sequence was non-labeled for use as competitor probe “PARAC536R”. We assumed that the melting characteristics of the probe and competitor were not affected by the presence/absence of the 5' label, and that signal discrimination remained the same using PARA536R / PARAC536R at 40% formamide in HB, though this assumption was not explicitly tested. PARA536R specificity was checked against the RDP II database, and was inclusive of *Paracoccus* but also several other *alphaproteobacteria* sequences including marine strains of *Rhodobacter*, *Rhodovulum*, and *Caulobacter*. Therefore, specific counts of *Roseobacter* using the highly specific ROSEOC536R probe are well supported, but specific counts of *Paracoccus* using the PARA536R probe may be overestimated due to lower specificity of PARA536R for *Paracoccus*, though the non-*Paracoccus* targets are still members of *alphaproteobacteria*.

Filters were prepared by gentle vacuum (-5 psi) filtration of 20 μ l sample in 1 ml 1X PBS onto 0.22 μ m nucleopore filters. The filters were placed temporarily on Whatman #4 filter paper pad saturated with 4% PFA solution in filtered seawater and cut in half with a sterile razor blade. Wet filter-halves were mounted sample-side-down on EtOH-cleaned, 0.1%-gelatin-coated slides, and allowed to completely air dry. The filters were gently peeled from the gelatin layer, leaving the filtered cells embedded partially in the gelatin layer.

The Hybridization Buffer (HB) consisted of 360 μ l 5M NaCl, 40 μ l 1 M TrisHCl (pH 7.5-8.0), 1.177 ml formamide (40% final concentration), 2.0 ml ddH₂O, and 2 μ l 10% SDS. Hybridizations were performed in 20 μ l volumes (18 μ l hybridization buffer (HB), 1 μ l ROSEO536R and 1 μ l ROSEOC536R (10 ng μ l⁻¹ final probe concentration)) by application of hybridization buffer directly to the embedded sample and covered with a cover slip. Hybridizations (overnight, 46°C) and washes (15 - 20 min. at 48°C) were performed as previously described by Stahl and Amann, 1991. Hybridization chambers consisted of 50 ml centrifuge tubes containing 2.0 ml of HB and a strip of Whatman filter paper. Sample slides were inserted into the centrifuge tubes and closed, and placed in an incubator maintained at 46°C. Rinses were performed after the elapsed hybridization period by gently immersing each slide into 50 ml centrifuge tubes containing wash buffer (WB) maintained at 48°C for 15 – 20 min. WB consisted of 460 μ l 5M NaCl, 1 ml 1 M Tris HCl (pH 7.5-8.0), 0.5 ml 0.5 M EDTA (pH 8.0), 50 ml ddH₂O, and 50 μ l 10% SDS. After the washing step, slides were gently immersed in ddH₂O in a staining jar. The slides were not rinsed with a squirt bottle after it was found that cell retention was

adversely affected by direct-stream rinsing. Slides were air-dried in the dark, treated with DAPI (4'-6-diamidino-2-phenylindole) (Hicks *et al.*, 1992) with 20 μ l of a 1.0- μ g/ μ l DAPI solution for 1 min. in the dark, and a final rinse in ddH₂O for 5 min. in a staining jar. Higher DAPI concentrations and/or longer staining times resulted in large DAPI background on emulsion-coated slides as DAPI diffused into the gelatin/emulsion layer and resisted rinsing. After airdrying, the samples were mounted in Citifluor and covered with a glass cover slip. This FISH protocol was adopted for all further FISH and STARFISH assays.

Fluorescent Microautoradiographic Microscopy

Microscopic counts were made using a Zeiss Axioplan 2 imaging microscope with a Zeiss α Plan-FLUAR 100X oil objective. DAPI counts were made with a UV lamp and filter set (Chroma Technology Corp.) exciter D360/40, emitter GG420. Cy3 counts were made with Endow GFP long pass emission filter set (Chroma Technology Corp.) exciter HQ470/40, emitter HQ500lp. Autoradiographic counts were made with transmitted incandescent light simultaneously with epifluorescent fields. A 100 x 100 μ m ocular counting grid was used to define fields for epifluorescent signal counting. For each microscopic field, Cy3 signals, DAPI signals, Cy3/emulsion exposures, and DAPI/emulsion exposures were counted and recorded. Ten random microscopic fields were counted on each slide for statistical purposes.

Cultured isolate testing

Four pure isolates were selected from our isolation program (see Chapter 3) for use in testing the STAR-FISH staining protocol. JT-01 (*Ruegeria atlantica*), a member of the *Roseobacter* clade and a CO-oxidizer; JT-16, a non-CO-oxidizing unidentified *Cytophaga* species, and JT-21, a CO-oxidizing *Cytophaga* member most closely related to *Cyclobacterium marinum*. Liquid cultures were sealed in a vial under zero-grade air (~20 ppm CO) and were incubated with injected ^{14}C -CO for a final dissolved [CO] of 40 nM for 1-4 days. The bottles were incubated on a rotary shaker table at 100 RPM for 24 - 48 hours. At the end of the incubation the sample was preserved by the addition of 0.22 μm -filtered formaldehyde to 4% final concentration. This concentration of fixative was found to be sufficient to stop uptake of labeled organic substances (Meyer-Reil, 1978). The entire sample was centrifuged for 10 min. at 9,000 rpm, and the pellet was transferred to a 2.0 ml centrifuge tube. The pellet was resuspended in 250 μl 1X PBS and 750 μl 4% paraformaldehyde solution, and placed in 4°C for two hours. The samples were centrifuged for 5 min. at 9,000 RPM, and the pellet was resuspended in a mixture containing 100 μl 4% PFA and 100 μl ethanol (98%). Samples were stored at -20°C when not in use.

STARFISH ^{14}C -CO incubation April 7, 2003

Coastal marine microbial assemblages were sampled at 0.5-meter depth at the Vineyard Sound sampling location (see Chapter 2) in a sterile glass bottle affixed to a pole. The sample was subdivided in the laboratory to perform several parallel analyses:

AODC or DAPI direct counting, rates of ^{14}C -CO oxidation to ^{14}C -CO₂, ^{14}C -CO-carbon uptake for microautoradiographic analysis, and whole-cell hybridization (figure 4.2).

Seawater sample (1.8 ml) was stained for 10 min by the addition of 0.2 ml 0.1% acridine orange (final concentration 0.01%) or alternatively for 1 min. with DAPI (1.0 µg/ml final concentration) within one hour of sample collection. The cells were gently vacuum filtered (5 mm Hg) onto a Sudan Black-stained 0.22 µm nucleopore membrane and enumerated by epifluorescence microscopy (*Hobbie et al.*, 1977)).

^{14}C -CO oxidation assay. (refer to Chapter 2 methods and materials)

Autoradiographic procedure.

Uptake of radioactivity by microorganisms was determined with a microautoradiographic technique based primarily on the “MARGE-E” procedure developed by Tabor and Neihof (1982), and the general procedures for autoradiography with liquid emulsions described by various authors (*Andreasen & Neilsen*, 1997). LM-1 hypercoat emulsion was the photographic medium used in our STARFISH assays (Amersham Pharmacia biotech, 1999). Each gram of LM-1 hypercoat emulsion contains the equivalent of 0.09g of silver (Ag) with an average crystal diameter of 0.2µm (when exposed and developed), 0.023g gelatin and 1% (w/v) plasticiser. An ethanol-cleaned 3” x 1” glass microslide was dipped in 0.1% gelatin at 46°C to create a gelatin sublayer (figure 4.1). After the gelatin layer was dry, the coated slide was immersed for 15 sec. in diluted LM-1 (1 part emulsion to 1 part 0.1% gelatin solution improved cell adhesion

compared to layering with emulsion only) (Amersham Pharmacia Biotech) maintained at 46°C under darkroom conditions (Kodak safelight filter GBX-2 placed 1 m away from the workspace). Later, a NightVision™ head-mounted IR illumination system was used in lieu of the safelight when manipulating unexposed emulsion-coated slides so as to further reduce autoradiographic background. The slide was drained horizontally for 20 sec. to produce a thin emulsion film. The emulsion was allowed to dry in the dark for 5 min. at room temperature.

Initial microautoradiographs showed large incidental radiographic exposures as a result of bioluminescent eucaryotic organisms present in the raw sample. To remove these organisms, sample water was 10.0 µm filtered. One hundred ml of 10 µm- filtered sample water was sealed within a 120 ml glass serum vial, and injected with ^{14}C -CO to result in a dissolved [CO] of 20 nM. Subsamples were removed at 2 hr., 4 hr., 6 hr., and 24 hr. intervals and were fixed with formaldehyde to a final concentration of 4%. These subsamples were filtered onto 0.22 µm filters and transferred to emulsion coated glass microscope slides to undergo autoradiography, FISH, and DAPI staining. The wet sample filters were applied with the sample side placed on the emulsion film carefully to exclude bubbles and to avoid lateral movement of the filter against the semi-solid emulsion. The slides were placed in a light-tight slide box containing a packet of silicon desiccant and allowed to set for another 3 hr., following the manufacturer's recommendations. After the emulsion was completely dry, the wooden light-tight slide box was wrapped in aluminum foil and placed at 4°C for radiographic exposure.

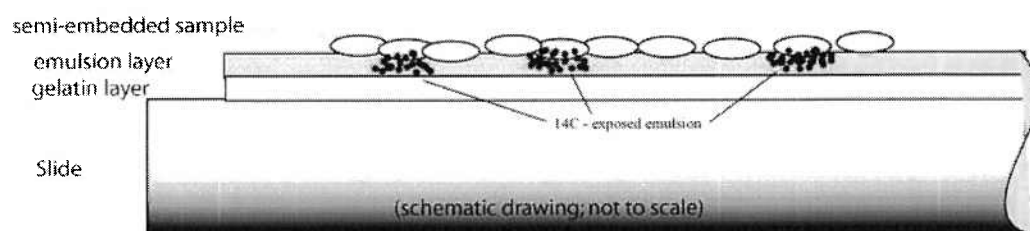


Figure 4.1: Side view of STARFISH cell adhesion protocol. Cells are partially embedded in the autoradiographic gelatin / emulsion mixture, so that silver grain exposure does not occlude fluorescence signal by either DAPI or Cy3 label. The gelatin sub-layer aids in cell retention throughout the process to within 90% of direct counts.

After the emulsion slides had been exposed for 1-3 days, they were developed using a standard photographic procedure. In the dark, slides were immersed in a staining jar containing Kodak GBX developer at 20°C (ambient lab temperature) for 5 min. They were transferred to 0.5% acetic acid in de-ionized water as a stop solution for 30 sec., and fixed for 10 min. in Kodak GBX fixer solution. The developer and fixer solutions were diluted per manufacturer's directions. The slides were rinsed for 20 min. in gently flowing tap water in a de-staining tray. After air-drying, the filter was gently peeled away from the emulsion film while gently breathing onto the backside of the filter. Attempting to peel the off the filter without humidifying resulted in tearing of the filter or removal of the emulsion film from the slide. Cells were dehydrated in an ethanol series (50%, 80%, 98% aqueous ethanol, v/v, 3 min. each) (*Stahl & Amann*, 1991). The slides may be stored at room temperature indefinitely, or FISH/DAPI processed immediately. Developed emulsion slides are FISH- and DAPI stained as previously described.

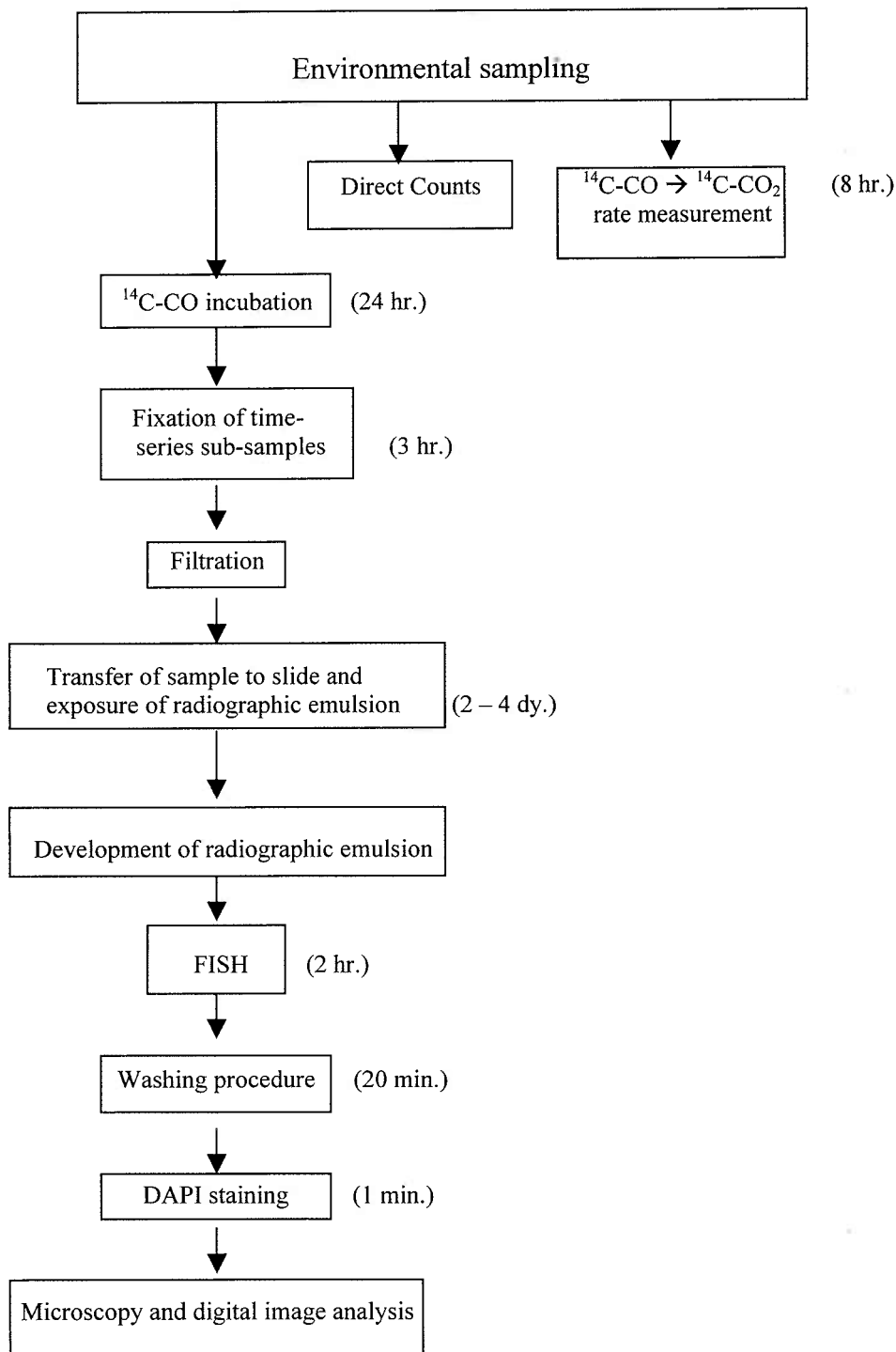


Figure 4.2: Order of staining operations for STARFISH triple-labeled samples. Times required for each step are included parenthetically. This sequence was found to minimize background silver grain exposure and DAPI absorption by the emulsion layer.

RESULTS and DISCUSSION

Roseobacter and *Paracoccus* are phylogenetically closely related genera within the alpha subgroup of the *Proteobacteria*, therefore, fine scale discrimination between these two sister groups using oligonucleotide probes targeted to the same region of 16S-rDNA necessarily must occur under strict stringency conditions. A high formamide concentration in combination with non-labeled competitor probes during whole-cell hybridization enabled differentiation between these sister groups. One mismatch to the ROSEO536R probe sequence was found after searching all non-target eubacterial and archaeobacterial 16S-rDNA sequence data accessible through the RDPII (<http://rdp.cme.msu.edu>) and Genbank (<http://www.ncbi.nlm.nih.gov>) databases. A competitor probe ROSEOC536R recognizing this single and consistent mismatch was used in hybridization experiments with natural samples in conjunction with probe ROSEO536R to maximize probe specificity of ROSEO536R by blocking this binding site of those species containing the single mismatch (*Amann et al.* 1990, *Brinkmeyer et al.*, 2000). The use of the ROSEO536R probe and competitor ROSEOC536R, and PARA536R and competitor PARAC536R, allow separate enumeration of *Roseobacter* and *Paracoccus*, collectively the marine alpha group, in natural samples.

All hybridizations were performed at a uniform temperature. Several of the taxon-specific probes reported by Brinkmeyer *et al.* (2000) exhibited attenuation of fluorescent signal at the higher formamide concentrations necessary to achieve optimal stringency at 46°C, and optimal formamide concentrations for ROSEO536R and ROSEOC536R were reported as 15-18% at 50°C. In following these recommendations,

however, we found the opposite; fluorescence by target type cells hybridized at their modified temperature and formamide concentrations was barely visible and impossible to count. Therefore, the optimal hybridization conditions for this study were re-evaluated. Optimal formamide concentration for high probe specificity at a 46°C hybridization temperature was determined by a formamide series with pure cultures (figure 4.3) and mixed cultures (figure 4.4) of *Roseobacter* and *Paracoccus* cells, and visualization of fluorescent signal brightness in natural samples. In FISH hybridizations using only ROSEO536R without the competitor probe, we found that 40% formamide in the hybridization buffer created the stringency required to discriminate between *Roseobacter*-clade cells, and closely related cells having the single mismatch at *E.coli* position 545 (figure 4.3). We decided that the ROSEO/ROSEOC combination in combination with the formamide concentration would produce clearer and more consistent discrimination of target from non-target organisms.

General staining with DAPI remained as the last step (refer to figure 4.2). We found that pre-staining the STARFISH time-series subsamples with DAPI or acridine orange prior to emulsion exposure produced a large autoradiographic background, probably due to residual fluorescence of the fluorochrome after light exposure. DAPI concentration and staining time was further modified from literature values. Rapid diffusion of DAPI into the gel/emulsion layer and its resulting resistance to rinsing produced a strong fluorescent background in the microscopic field, making total cell counts difficult and inaccurate. One minute of direct staining using 1 µg/ml DAPI was sufficient to stain the cells without excess retention of DAPI in the emulsion layer.

To calculate the CO that could potentially be oxidized by target group cells, the relative densities of these groups in natural samples were multiplied by specific CO oxidation rate measurements of the coastal CO oxidizing isolates (Chapter 3). Because these specific rate measurements were conducted at 19 – 20°C (laboratory ambient temperature), and the STARFISH experiment was conducted at *in situ* temperature (6°C, March, 2003), a parallel CO oxidation experiment was conducted at two temperatures to determine the Arrhenius correction required. The rate of overall CO oxidation in the natural sample at 19°C was 0.077 nM hr⁻¹ greater than at 6°C, (20% increase), and the dissolved [CO] in the 6°C incubation was 21% greater than at 19°C. Both samples had been stripped of natural CO before incubation with ¹⁴C-CO, and injected with precise and equal volumes of labeled CO. When the rate coefficient k_{co} was calculated from these values for rate and [CO], we determined that k_{co} was 54% higher at 19°C than at 6°C (figure 4.5). The rate coefficients and specific rates of CO oxidation that were measured for CO oxidizing isolates determined at 20°C were therefore adjusted for lower field temperature by application of the Arrhenius equation, $k = A e^{-E_a/RT}$, where the frequency factor A was calculated for the rate coefficient for each isolate at 20°C, the universal gas constant $R = 8.314 \text{ joules deg}^{-1}\text{mol}^{-1}$, and $T = \text{temperature in Kelvins}$. The activation energy $E_a = 26.13 \text{ KJ mol}^{-1}$ was calculated from the slope of a best fit line in a graph of $\log k_{co}$ versus $1/T$ from the two temperature CO oxidation experiment ($E_a = \text{slope} \times -2.303R$) (figure 4.6) (Daniels & Alberty, 1967).

Figure 4.3: Formamide series. 40% formamide within the hybridization buffer (HB) was necessary to permit discrimination between *Roseobacter* family cells from *Paracoccus* having a single consistent mismatch from ROSEO536R, without use of a competitor probe having exact sequence compliment to the mismatch. Cy3 fluorescence signal in *Paracoccus* (right side in each pair) is absent at 40% formamide in the hybridization buffer (negative images).

2

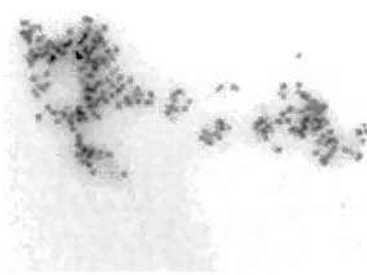


P. dentrificans

**30%
formamide**



R. dentrificans



R. dentrificans

P. dentrificans

**40%
formamide**



R. dentrificans

P. dentrificans

**25%
formamide**

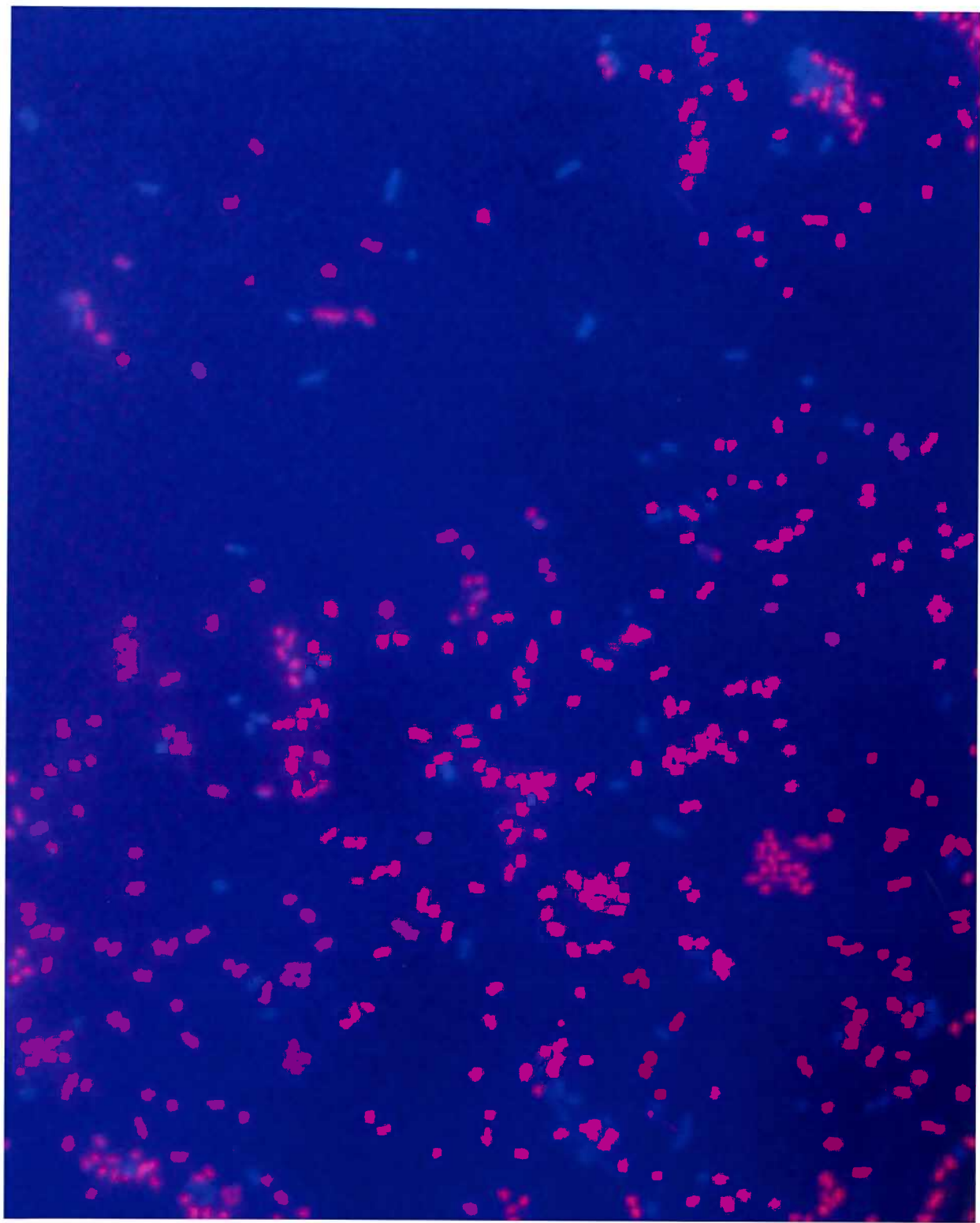


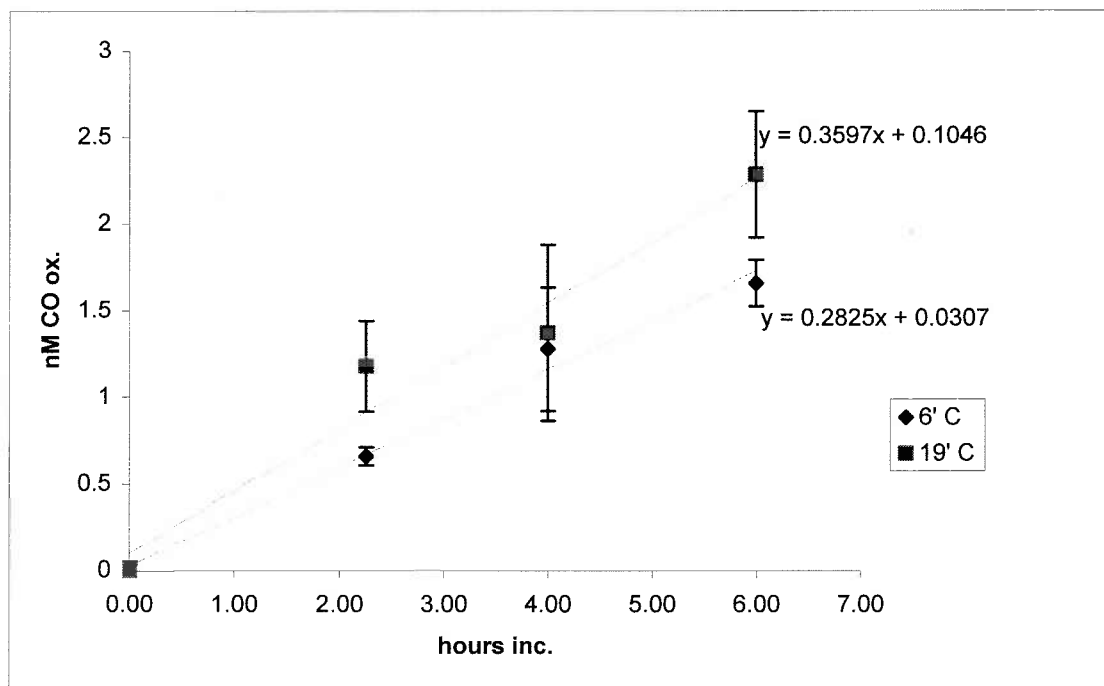
R. dentrificans

P. dentrificans

**35%
formamide**

Figure 4.4: Mixed binary culture of *Roseobacter litoralis* and *Paracoccus denitrificans*. Digital overlay of microscopic fields viewed with UV to show DAPI stained cells, and 500 nm long pass filter to view Cy3-labeled target cells. *Roseobacter* cells are illuminated by ROSEO536R used in combination with the competitive probe ROSEOC536R and at 40% formamide stringency and appear pink, while *Paracoccus* stained only with DAPI appear blue. This demonstrates fine-scale discrimination between cells having exact and single-mismatch sequences to the ROSEO probe.





temp.	rate (nM/hr)	[CO](nM)	k (hr ⁻¹)
19	0.360	4.332	0.083
6	0.283	5.270	0.054

Figure 4.5: Two-temperature CO oxidation test. CO oxidation in natural seawater progressed at a lesser rate at *in situ* temperature (6°C) than at laboratory ambient temperature (19°C). Rate coefficients for pure isolates determined at 20°C require correction before application to *in situ* rate calculations.

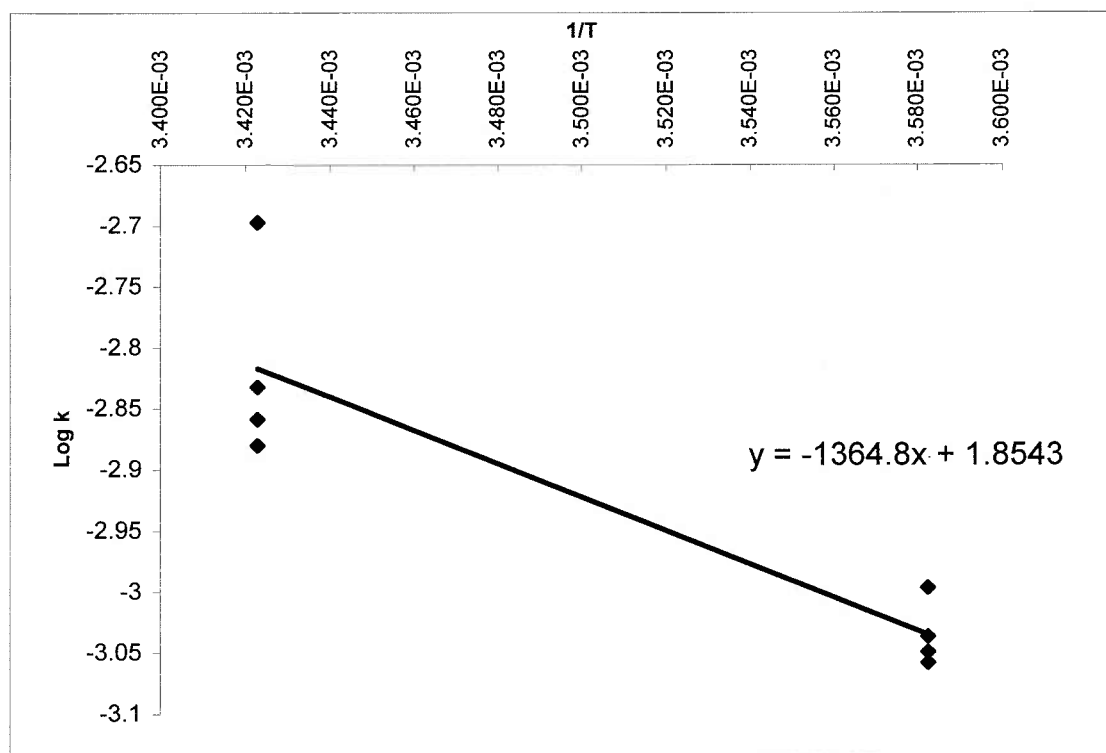


Figure 4.6: Graph of $\log k_{co}$ versus $1/T$ for CO oxidation by *in situ* microbial assemblage, from which the Arrhenius activation energy E_a was calculated from the slope.

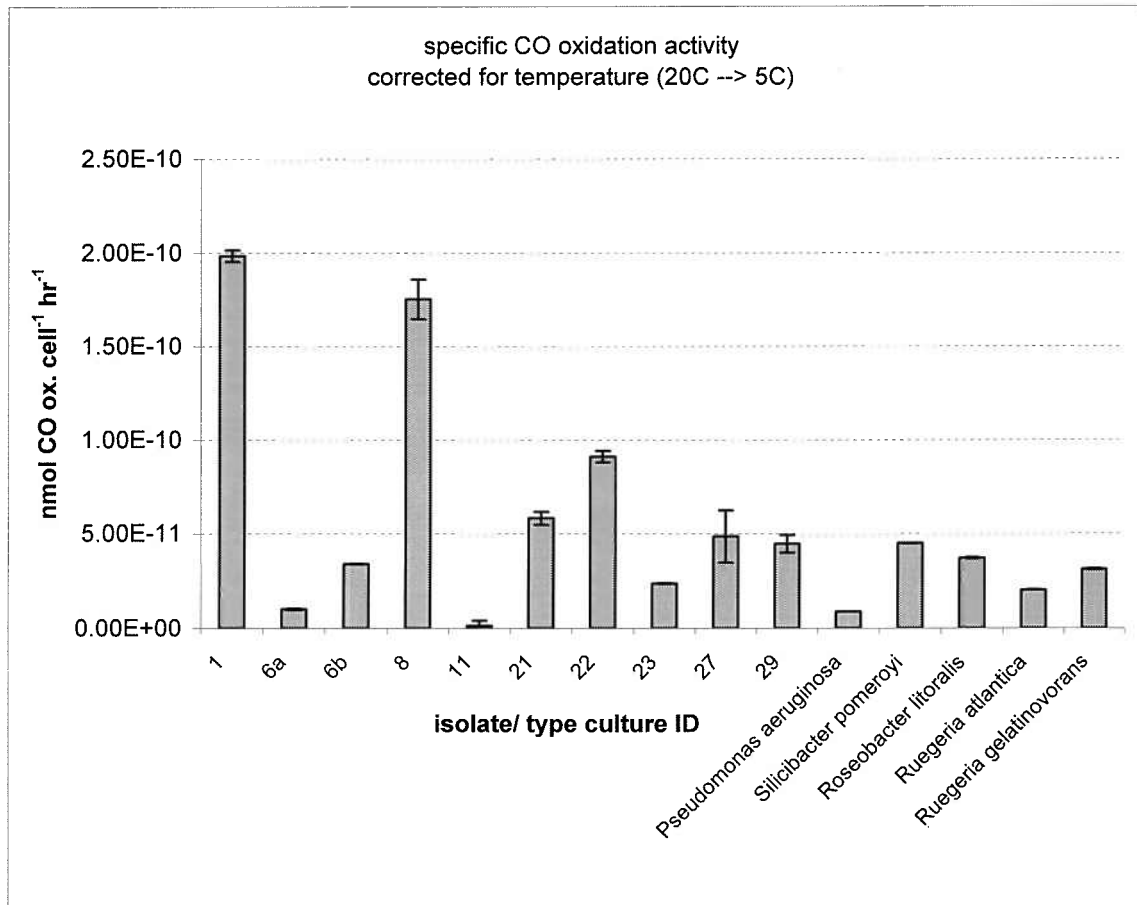


Figure 4.7: Specific CO oxidation activity for active CO oxidizing isolates and type cultures, corrected for temperature in rate coefficients k_{co} determined at 20°C for pure cultures and applied to incubations at 5°C.

The temperature-adjusted rate constants for the pure culture isolates were approximately 35% lower when applied to 5°C (figure 4.7) than at 20°C (figure 3.11, 3.15).

Two factors affect the value of k_{co} : the temperature during incubation and dissolved [CO] that varies inversely with temperature. The pre-and post-correction k_{co} values yield $Q_{10} = 1.2$, a departure from the general rule that reaction rates approximately double with every 10° increase in temperature ($Q_{10} = \sim 2$). If we postulate that CO behaves as a non-gaseous solute (concentration non varying with temperature), and adjust k_{co} only for temperature effect, then Q_{10} becomes 1.73, much nearer the rule-of-thumb value. Since both temperature and its effect on dissolved [CO] are in fact operative in this experiment, we will accept the low value of Q_{10} and use the k_{co} values corrected for both variables in our rate calculations.

The STARFISH experiment began with a sample collected from the Vineyard Sound collection site (figure 2.1) on April 7, 2003. The gross rate of CO oxidation (0.0882 nM CO oxidized hr⁻¹, $k_{co} = 0.021$ hr⁻¹) was determined via ¹⁴C-CO oxidation assay (figure 4.8) begun within 1 hour of sampling. Microscopic direct counts of sample water showed a total cell density of $1.17 \times 10^6 \pm 2.25 \times 10^4$ cells/ml (mean \pm SEM, n=10). After the STARFISH incubation sub-samples had been triple labeled and mounted, microscopic counts of cells in the same field under four different regimes of illumination per field were recorded. The UV 552/568 (absorb/emit) filter set illuminated cells that had reacted with Cy3-labeled probes. Separate slide sets were prepared to enumerate *Roseobacter* (figure 4.9) and *Paracoccus* separately, by their respective probe sets.

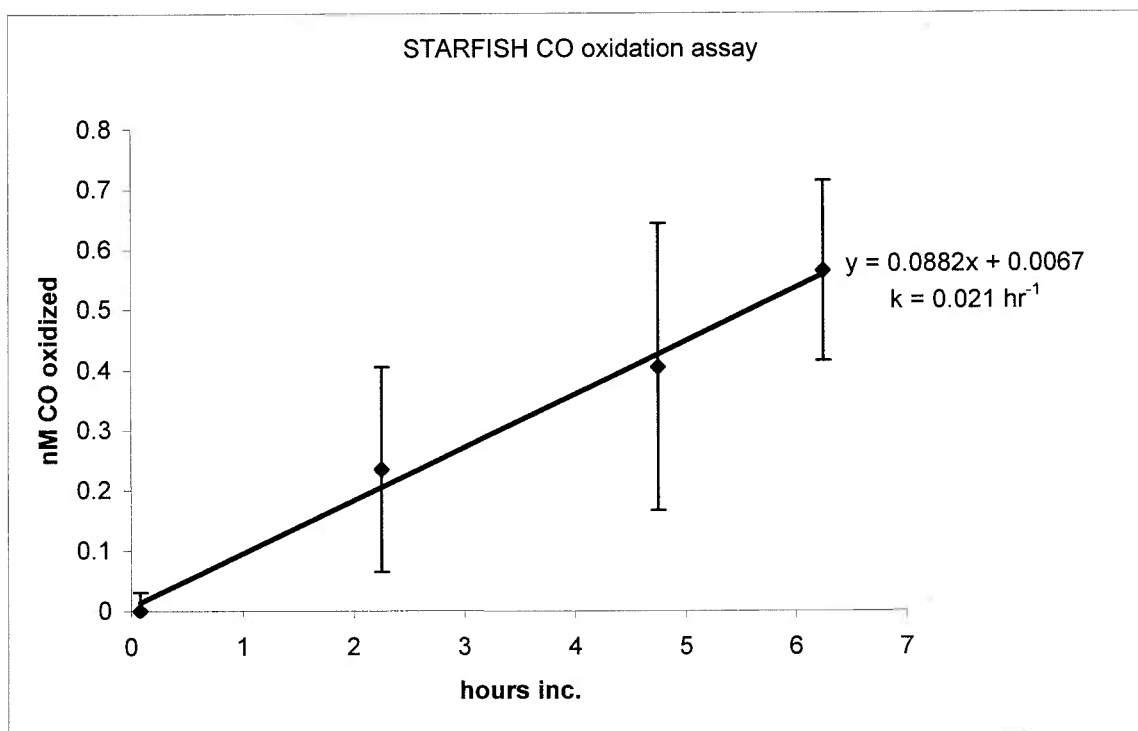
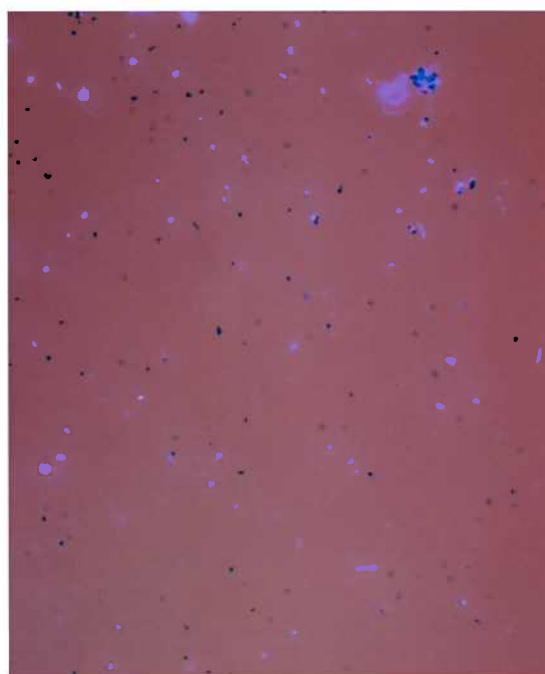
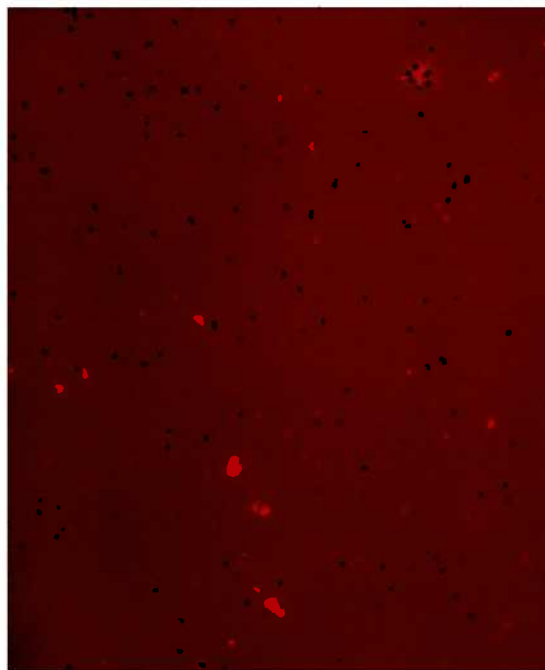
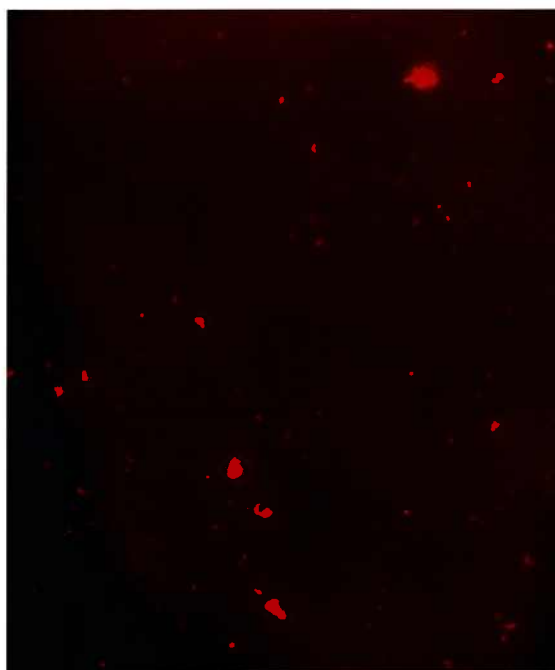


Figure 4.8: CO oxidation assay for STARFISH experiment. Four time-point incubation conducted at *in situ* (6°C) temperature, 25 ml volumes of natural sample, n=2 for each time-point.

Figure 4.9: Representative counting field for STARFISH enumeration of CO metabolizing *Roseobacter* cells in natural coastal sample. Cells fixed after 6 hours of incubation with ^{14}C -CO.
Upper Left: Cy3 signal from *Roseobacter* specific staining.
Upper Right: Cy3 and transmitted light image of *Roseobacter* and ^{14}C -containing cells.
Lower Left: DAPI stained cells. (total)
Lower Right: DAPI and transmitted light image.



Simultaneous use of UV and transmitted light allowed visualization of Cy3-labeled cells that incorporated label from ^{14}C -CO. The UV 360/420 filter set allowed visualization of all cells stained with DAPI and a total cell count. A fourth enumeration was made of DAPI-stained cells that incorporated label from ^{14}C -CO in each microscopic field.

Total cell counts on STARFISH slides were compared with direct filter counts to establish quantitative cell retention during the STARFISH preparations. Within experimental error, cells that were mounted on slide emulsions from filters and undergoing the multiple immersions and rinses in the STARFISH protocol were quantitatively retained on the slides. Early experiments possessed only 60% - 70% cell retention after STARFISH treatments, until rinsing under flowing water was replaced with gentle static immersion in rinse fluid to avoid the mechanical removal of cells from the emulsion layer.

In situ cell density for each probe-targeted group was estimated by calculating the ratio of Cy3 signals to DAPI signals per microscopic field (n=10 per light field (4), per slide (2), per time-point (4)). This ratio was multiplied by DAPI direct count of the sample, resulting in a group-specific cell density for *Roseobacter* and *Paracoccus* cells in the natural sample (figure 4.10). The marine alpha group composed 45.7% of the total microbial assemblage at the time of sampling (+ 2 hours), and increased to 65% of the assemblage after 24 hours of ^{14}C -CO incubation at 5°C. Approximately 10% of Cy3-labelled *Roseobacter* cells also had an autoradiographic signal attached, and nearly 20% of Cy3-labelled *Paracoccus* were active in ^{14}C -CO metabolism.

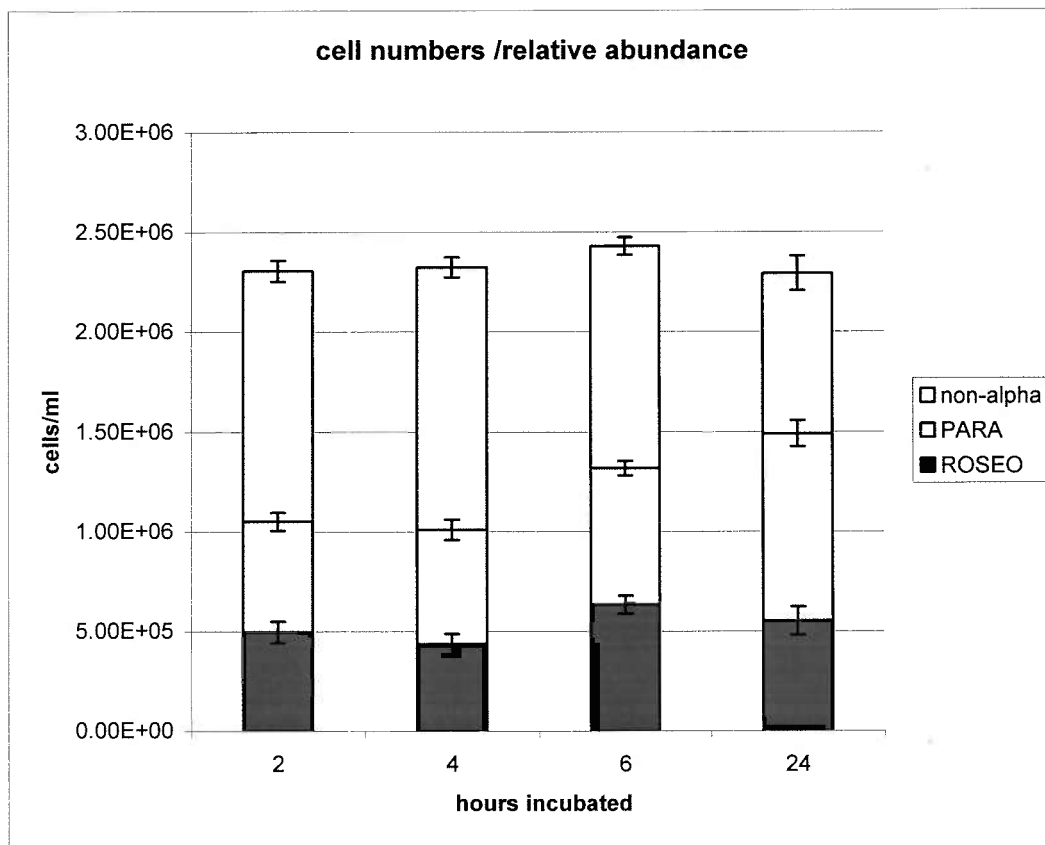


Figure 4.10: Differential cell counts in STARFISH preparations, (scaled). *Roseobacter* Cy3 signals (bottom), *Paracoccus* Cy3 signals (middle), and difference (total DAPI signal minus Cy3 signal) (top)

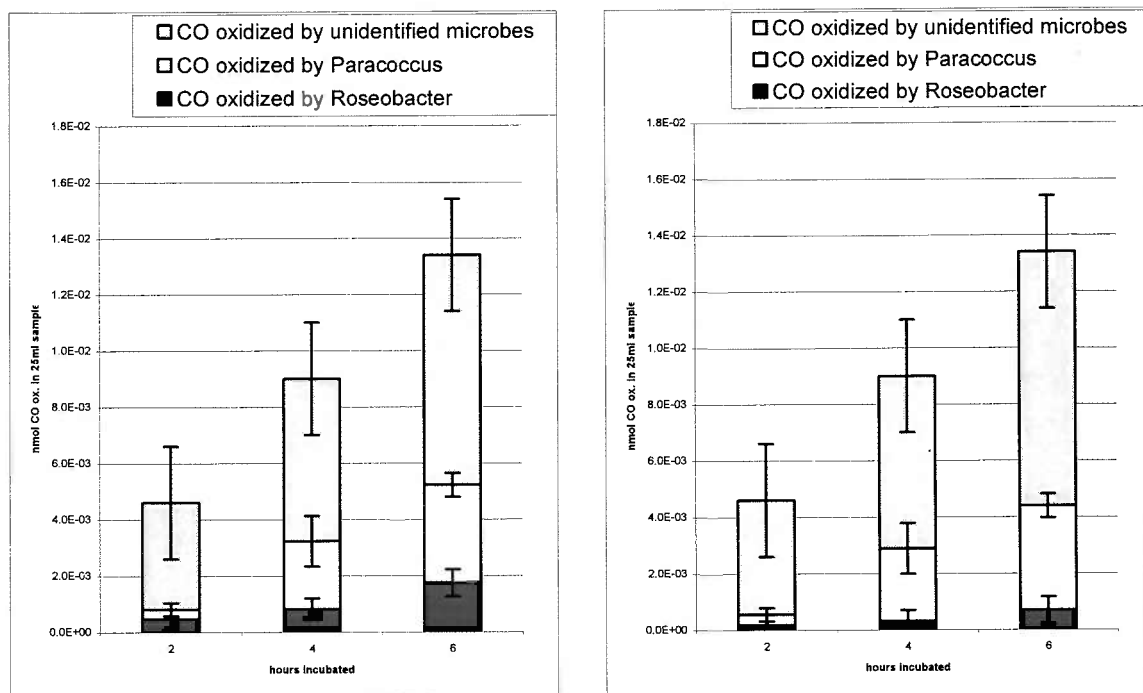


Figure 4.11: CO oxidized by target groups. *Roseobacter*(bottom), *Paracoccus* (2nd from bottom), and unaccounted residual CO oxidized (top). Graph on left shows the maximum CO oxidation values based on isolates with greatest cell specific activities for *Roseobacter* (JT-08) and *Paracoccus* (JT-01). The graph on the right shows the average CO oxidation values based on average cell specific CO oxidation activities for *Roseobacter* (6 spp.) and *Paracoccus* (JT-01, 2 growth conditions)

The ratio of Cy3- and ^{14}C -positive cells to DAPI-positive cells on the STARFISH slides, multiplied by cell density determined by DAPI direct counts on separate slides, yielded the relative density of active CO-metabolizing cells belonging to *Roseobacter* or *Paracoccus* groups. These activity- and group-specific cell densities (cells ml^{-1}) were multiplied by the specific CO-oxidation activity term ($\text{nmolCO oxidized cell}^{-1} \text{ hr}^{-1}$, corrected for temperature difference), to estimate the molar amount of CO oxidized that could be attributed to *Roseobacter* and *Paracoccus* organisms.

A maximum estimate of CO oxidation activity attributed to *Roseobacter* and *Paracoccus* was determined by using the cell specific CO oxidation activities of the isolates exhibiting the highest specific CO oxidation activities of each group (JT-01, JT-08; figure 4.7). A second, more conservative estimate was calculated using average values of CO oxidation activity by *Roseobacter* and *Paracoccus* (figure 4.11). The sole CO oxidizing *Paracoccus* strain in our study, JT-01, oxidized CO at a rate two-fold greater than other isolates except JT-08, a *Roseobacter* strain. Since the average specific CO oxidation activity value that can be calculated for *Paracoccus* pertains to only one strain grown in two different nitrogen-containing media ($n=2$), the average CO oxidation attributed to *Paracoccus* is large since the maximum and average values are similar. The average activity for *Roseobacter*, however, is more certain as six isolates and type strains were confirmed as CO oxidizing organisms ($n=8$) with only one having an anomalously high specific rate.

Up to 40.7% of total CO oxidation at this sampling can be attributed to marine alpha group organisms isolated from the environment and their specific metabolic rates that we obtained in Chapter 3. After 2 hours of incubation with ^{14}C -CO, there is a low density of ^{14}C -labeled cells of any variety, suggesting that there is a time dependence on label uptake before intracellular ^{14}C reaches a threshold activity sufficient to create an autoradiographic signal. ^{14}C -positive signal ratio, the relative numbers of *Roseobacter* and *Paracoccus*, and thus, the CO-oxidation activity attributed each group, vary only slightly with time after 4 hours of ^{14}C -CO incubation. After six hours of incubation with ^{14}C -CO, active CO-metabolizing cells belonging to the *Roseobacter* group account for 5.2% – 13.1% of total CO oxidation, and *Paracoccus* CO-metabolizing cells account for 25.8% – 27.6%. Thus, 31% - 40.7% of total *in situ* CO oxidation is attributed to the marine alpha group collectively. This value is less than the initial estimate that was based on rate coefficients, made in Chapter 3 (50%), but is still a substantial fraction of the total activity.

The marine alpha group organisms are emerging as numerically dominant and metabolically versatile in various marine habitats. The ability of *Roseobacter* and its sister taxon *Paracoccus* to oxidize photolytically produced CO, and the large relative proportion of total *in situ* CO oxidation for which they are accountable has been hitherto unreported.

We have in pure culture several microorganisms isolated from coastal seawater that are not closely related to the marine alpha group but are capable CO-oxidation at environmentally relevant levels. These have representatives in *betaproteobacteria*,

gammaproteobacteria, and the phylum *Bacteroidetes* (Chapter 3). The relative proportion of total CO oxidation performed by these groups cannot yet be resolved in natural samples because we have not enumerated these component groups via the STARFISH assay. By using extant general oligonucleotide probes for these phylogenetic groups, it is a viable and practical endeavor to enumerate these groups in natural waters and determine their relative contributions to the total CO-metabolism occurring there. With use of group-specific probes in STARFISH, the efficacy of this assay has been demonstrated for quantifying a numerically and metabolically important phylogenetic group contributing to a natural chemical transformation that occurs in marine environments. A more complete picture of the CO oxidizing marine community may be obtained when more CO-oxidizing taxa have been quantified and included in the CO oxidation budget.

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SUMMARY and CONCLUSIONS

This project has explored and described the biodiversity of organisms that contribute to a specific chemical transformation occurring in natural waters, one that mediates the flux of CO to the atmosphere and returns carbon to biological cycling. State-of-art methods in molecular biology in a novel application have resulted in a richer understanding of this important process in the ocean. Biological involvement in CO oxidation in natural seawater has long been acknowledged in studies of trace gasses in the ocean and atmosphere, but the identities of CO oxidizing microbes have remained obscure. A major thrust of our study has been to determine if there exists a specialized group of microorganisms that is responsible for CO metabolism at environmental concentrations, or if CO at environmental concentrations is oxidized and/or consumed by several members of the microbial assemblage present in the environment. We have isolated several pure strains of microorganisms from natural coastal seawater with a novel technique that involves macroautoradiography to target, isolate, and quantify microbial colonies that incorporate label from a ^{14}C -CO-containing atmosphere, and have determined the cell-specific CO-metabolic rates of these organisms in pure cultures in laboratory experiments. Furthermore, we have provided the first definitive identification of some of the diverse CO metabolizing microorganisms that exist in a coastal environment. This research has begun to illuminate the rich phylogenetic diversity of microorganisms that utilize an inorganic carbon substrate in coastal waters.

Objective 1: Isolation and Culture of marine CO oxidizing microorganisms.

- 1) Thirty CO-tolerant isolates result from the CO incubation program.
- 2) Nine isolates are positive for CO oxidation at environmentally relevant activities, at cell-specific activities ranging from $3.0 \times 10^{-11} - 2.3 \times 10^{-10}$ nmol CO oxidized cell⁻¹ hr⁻¹ at 20°C ($2.37 \times 10^{-11} - 1.75 \times 10^{-10}$ nmol CO oxidized cell⁻¹ hr⁻¹ at 5°C).
- 3) In 8 of 9 isolates, nitrogen source had no effect on CO oxidation activity. JT-08 (a *Roseobacter* group organism) is the exception, where CO-oxidation activity with NH₄⁺ was ~50% of activity with NO₃⁻.
- 4) All isolates resulting from the incubation program are able to grow in heterotrophic media, and all incorporate ¹⁴C from CO strongly while growing in heterotrophic conditions.
- 5) ¹⁴C- incorporation was weak or absent in most CO-oxidizing isolates while in oligotrophic growth conditions. JT-01 (a *Paracoccus* group organism) is the exception, with strong label incorporation in all three growth media (2216 broth, NO₃⁻ mineral media, or NH₄⁺ mineral media)

Objective 2: Identification and phylogeny of CO oxidizing isolates based on small subunit ribosomal gene sequence.

- 1) The three most active CO oxidizing organisms isolated in our incubation program are phylogenetically clustered within the marine alpha group (*Roseobacter* and *Paracoccus*) of the *alphaproteobacteria* (JT-01, JT-08, JT-22).
- 2) Several type cultures of marine alpha organisms are positive and active CO oxidizers (*Ruegeria atlantica*, *Silicibacter pomeroyi*, *Ruegeria gelatinovora*, and *Roseobacter litoralis*), but CO oxidation is not a characteristic phenotype of *Roseobacter*, *Paracoccus*, or the marine alpha group.
- 3) The diversity of the CO-oxidizing isolates resulting from this program is broad, with representatives in the the *alphaproteobacteria*, *betaproteobacteria*, *gammaproteobacteria*, and Phylum *Bacteroidetes*.

Objective 3: STARFISH assay to enumerate CO oxidizing organisms in natural samples

- 1) The marine alpha group composed 45.7% of the total microbial assemblage in a coastal environment in early spring.
- 2) Oligonucleotide probes specific for *Roseobacter* and *Paracoccus*, in combination with microautoradiography, reveal that the marine alpha group contributes 31% - 41% of total *in situ* CO oxidation, based on Cy3 probe- and ¹⁴C-labeled cell counts in natural samples.

Future Research Directions

STARFISH with 16S-rDNA probes spanning the diversity of CO-oxidizing isolates

Our protocol to enumerate CO-metabolizing prokaryotic groups with environmentally relevant CO-metabolic activities on a per-cell basis in coastal waters and calculate their relative contributions to total *in situ* CO metabolism has been productive and successful in assigning the proportion of a natural chemical conversion in seawater to specific microbial groups. However, other diverse seawater microorganisms from our isolation program also have demonstrated the ability to metabolize CO and are likely important contributors to total measured biological CO oxidation in coastal seawater. These organisms must be quantified in natural samples in the same manner to determine their relative importance to total *in situ* CO metabolism.

To fulfill this objective we will utilize STAR-FISH for simultaneous *in situ* identification, quantification, and determination of substrate uptake patterns of individual microbial cells within a natural microbial assemblage. We will perform STAR-FISH experiments with coastal seawater samples collected locally to target and quantify a variety of phylogenetic groups, including *Alteromonas*-like species (*Gammaproteobacteria*), *Halomonas*- and *Pseudomonas*-like species (*betaproteobacteria*), and *Cyclobacterium*-like species (phylum Bacteroidetes), which we currently believe contribute to *in situ* CO metabolism based on laboratory studies of pure cultures. The STAR-FISH approach additionally will indicate other phylogenetic groups performing CO metabolism that are as yet unknown to us and absent in our culture collection, limited only by our breadth and depth of probe utilization. The STAR-FISH

experiments in combination with existing specific metabolic measurements from pure cultures will allow simultaneous determination of relative abundance, relative importance to CO-consumption in coastal waters, and balancing the observed CO-consumption in bulk water samples. (Proposed, WHOI SeaGrant, WHOI proposal # BI11269 denied; Ocean Life Institute, WHOI proposal # BI11345 pending)

Seasonal monitoring of community structure

We will monitor the *in situ* community structure and CO metabolic rates at several sampling events throughout the year to determine seasonal shifts in abundance and activity of the major CO-metabolizing microbial groups, and how this relates to seasonal variability in CO bio-consumption that is observed in the coastal environment.

STARFISH in an oceanic environment

In an oceanic environment (BATS) during summer 1999 and early spring 2000, we monitored the diel variability of photolytically produced CO and its rate of microbial oxidation. While the question of which organisms are performing CO bio-oxidation in coastal surface waters is being resolved, the identities and relative abundances of the microorganisms responsible for *in situ* CO bio-oxidation in oceanic surface waters requires further investigation. There are many published studies concerning CO photo-production, *in situ* consumption, and atmospheric flux in oceanic surface waters, but none concerning the identity of the microbes responsible for oceanic CO bio-oxidation. Our objectives are to (a) apply our field-proven techniques for direct isolation and identification of microorganisms to surface waters of the oligotrophic open ocean, (b) determine specific CO-oxidation activities of all resulting isolates, (c) develop molecular

probes based on molecular phylogeny of resulting isolates and (d) apply STAR-FISH for simultaneous identification, quantification, and determination of substrate uptake patterns of individual microbial cells within an oceanic microbial assemblage. While CO-processes (production and consumption) have been measured on a gross scale in bulk seawater samples for nearly three decades in oligotrophic oceanic waters, this will be the first attempt to both quantitatively and qualitatively describe the organisms responsible for oceanic CO-metabolism, and illuminate the 'black box' regarding CO consumption in seawater that has been up to now overlooked in studies of ocean-atmosphere flux of trace gasses and carbon budget models in the oceanic environment. (proposed, Access to the Sea, WHOI proposal # BI11285 denied)

Amplification/probing of CO-DH genes

Carbon monoxide dehydrogenase (CO-DH) (*Meyer et al.*, 1986, *Kim & Hegeman*, 1983) is a key enzyme in energy metabolism of many described CO-metabolizing bacteria isolated from terrestrial sources. CO-DH serves three functions: (*a*) to feed electrons into the respiratory chain for electron transport phosphorylation, (*b*) to provide the carbon source as CO₂, and (*c*) to provide ATP and, by inverse electron transfer, NADH for CO₂ assimilation via the reductive pentosephosphate cycle. It is possible that CO-metabolizing organisms isolated from marine sources may also possess this enzyme. Primers and probes for CO-DH are being currently being developed (*Gary King, personal communication*) and may be adapted for use in detecting this gene in marine environments and in STAR-FISH to directly enumerate CO-metabolizing cells that also contain the genes for CODH.

APPENDIX A: Mineral media for ^{14}C -CO incubations

<u>6 salt base (Solution 1)</u>	<u>g/l dH₂O</u>
NaCl	20.0
MgCl ₂ · 6H ₂ O	3.0
MgSO ₄ · 7H ₂ O	6.0
KCl	0.5
NaHCO ₃	0.2
CaCl ₂ · 2H ₂ O	0.3
Autoclave to sterilize	

<u>Phosphate Solution 2</u> (autoclave separately from solution 1)	
K ₂ HPO ₄	17.5g/500 ml dH ₂ O

<u>Trace Element Solution 3</u>	<u>g/l dH₂O (modified Pfennig SL-8)</u>
ZnCl ₂	0.7
MnCl ₂ · 4H ₂ O	0.1
H ₃ BO ₃	0.62
CoCl ₂ · 6H ₂ O	0.19
CuCl ₂ · 2H ₂ O	0.17
NiCl ₂ · 6H ₂ O	0.024
Na ₂ MoO ₄ · 2H ₂ O	0.036
Na ₂ SeO ₃	0.02
Adjust to pH 6.5 with HCl, 0.22 filter sterilize and refrigerate	

<u>Vitamin Solution 4</u>	<u>g/500ml dH₂O</u>
Niacin	5 mg
Biotin	2 mg
B ₁₂ (cobalamine)	5 mg
Do not autoclave. 0.22 µm filter sterilize and refrigerate	

<u>Nitrate Solution 5</u>	
NaNO ₃	65 g / 250ml dH ₂ O
Autoclave to sterilize	

<u>Ammonium Solution 6</u>	
(NH ₄) ₂ SO ₄	50 g / 250ml dH ₂ O
autoclave to sterilize	

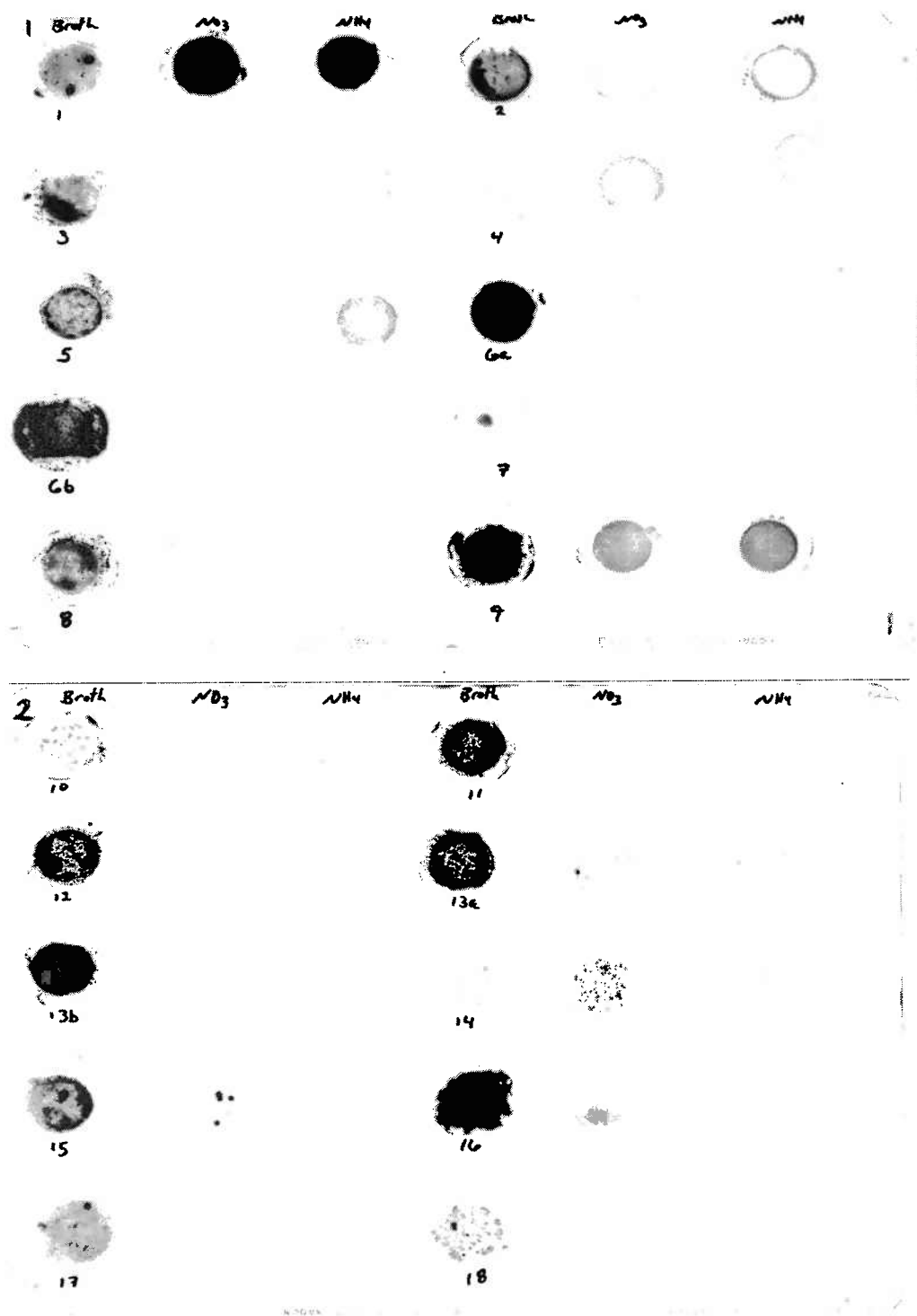
FINAL SOLUTION

Add 1.0 ml of Solutions 2, 3, and 4 to 1.0 l 6-salt base (Solution 1) after cooling. For differential nitrogen media, add 1 ml of either solution 5 or solution 6.

Final concentrations:

PO ₄ ³⁻	0.245 mM
NO ₃ ⁻	3.06 mM
NH ₄ ⁺	3.04 mM

APPENDIX B: autoradiographs – second round, purified isolates



3 Broth

neg

NTM

Broth

neg

NTM



21

22



23

24



25

26



27

28

4 Broth

neg

NTM

Broth

neg

NTM



29

30

X

e.coli

e.coli

e.coli



X

Broth control

Broth control

APPENDIX C: Characterizations of CO oxidizing isolates resulting from this study are excerpted from Bergey's Manual of Determinative Bacteriology, 9th ed.

Genus *Roseobacter* are aerobic and phototrophic Purple Bacteria that grow heterotrophically in aerobic conditions but also synthesize Bacteriochlorophyll *a* in the presence of oxygen. They form intracellular membranes and have reaction center complexes similar to those of other purple bacteria. *Roseobacter* cells are straight rods having 0.6 – 0.9 µm diameter and 1.0 – 2.0 µm length, are motile in liquid media and have polarly arranged flagella. Thiamine and niacin are required for growth, as well as Na⁺, biotin and nicotinic acid. The optimal pH for growth ranges from 7.0 – 8.0, and the optimum temperature ranges from 20 – 30°C. They are susceptible to chloramphenicol, penicillin, tetracycline, streptomycin, and polymyxin B.

Paracoccus are spherical cells (0.5 – 0.9 µm in diameter) or short rods (0.9-1.2 µm long), and occur singly, in pairs, or in clusters. No resting stages are known. Cells stain Gram negative. They are non-motile and aerobic, having a strictly respiratory type of metabolism; anaerobic growth can occur if nitrate, nitrite or nitrous oxide are present as terminal electron acceptor. Nitrate is reduced to nitrous oxide and molecular nitrogen under anaerobic conditions. One species (*P. dentrificans*) can grow either autotrophically with H₂ and CO₂ or heterotrophically with a wide variety of organic compounds as sole carbon sources, though this species is not halophilic. A second species (*P. halodenitrificans*) is not capable of autotrophic growth but is halophilic (requires at least 3% NaCl for growth). Optimum temperature is 25 – 30°C. They occur in soil and presumably in natural and artificial brines.

Genus *Halomonas* are rod-shaped or pleomorphic. Rods are generally 0.6-0.8 μm x 1.6-1.9 μm . Elongated, flexuous filaments may be formed under certain conditions. Cells stain Gram negative. Motile or non-motile; motility is by means of several unsheathed lateral of polar flagella. They possess mainly a respiratory type of metabolism with oxygen as the terminal electron acceptor. Growth of some strains may occur anaerobically with nitrate. Colonies are white to yellow, never red. Carbohydrates, amino acids, and some polyols can serve as sole carbon sources in mineral media. Catalase and oxidase positive. Most strains reduce nitrate to nitrite. Chemoorganotrophic. Ammonium sulfate can serve as sole nitrogen source. Halotolerant, able to grow in NaCl concentrations ranging from 0-0.5-20% or more. Strains have been isolated from a solar salt facility, from a saline lake in Antarctica, and from an estuary in New Hampshire.

Genus *Cyclobacterium* contains only a single species, *Cyclobacterium marinum*. The genus consists of circle-shaped (ringlike) and horseshoe-shaped cells with an outer diameter of 0.8-1.5 μm and a cell width of 0.3-0.7 μm . The cells have rounded (never tapered) ends. Coils, spiral forms, and some straight rods occur less frequently; filamentous or pleomorphic cells are rare. Stain is Gram-negative. Cells are encapsulated. They are non-flagellated and non-motile. No gas vesicles occur. No resting or life cycle stages occur. Optimum growth occurs at 20-25°C in media containing seawater or 3.0% NaCl. Aerobic, having a strictly respiratory type of metabolism. Convex, mucoid, opaque, smooth, small (<2-mm) pink colonies grow on modified Zobell marine agar or tryptone-glucose-yeast extract agar containing 3% NaCl.

Oxidase and catalase positive. Habitat is marine environments. Cells are resistant to chlortetracycline, kanamycin, penicillin G, streptomycin, and sulfmethoxazole/trimethoprim.

Genus *Pseudomonas* are straight or slightly curved rods, but not helical, 0.5 – 1.0 x 1.5-5.0 μm . They do not produce prosthecea and are not surrounded by sheaths. No resting stages are known. Cells stain Gram negative. Motility occurs by one or several polar flagella; they are rarely non-motile. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor; in some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. Most, if not all, species fail to grow under acidic conditions (pH 4.5). Most species do not require organic growth factors. Oxidase positive or negative. Catalase positive and chemoorganotrophic; some species are facultative chemolithotrophs, able to use H_2 or CO as energy sources. Widely distributed in nature. Some species are pathogenic for human, animals, or plants.

APPENDIX D: aligned 16S-rDNA sequences of CO oxidizing environmental isolates resulting from Chapter 3 isolation experiments (*E. coli* numbering).

	451	461	471	481	491	500
JT-01-b						
	
	501	511	521	531	541	550
JT-01-b						
	GA	GGGGUUAGCG
	551	561	571	581	591	600
JT-01-b						
	UUGUUCGGAA	UUACUGGGCG	UAAAUCGCGC	GUAGGCGGAU	UGGAAAGUUG	
	601	611	621	631	641	650
JT-01-b						
	GGGGUGAAAU	CCCGGGGCUC	AACCUCGGAA	CUGCCUCCAA	AACUCCCAGU	
	651	661	671	681	691	700
JT-01-b						
	CUUGAGUUCG	AGAGAGGUGA	GUGGAACUCC	GAGUGUAGAG	GUGAAAUUCG	
	701	711	721	731	741	750
JT-01-b						
	UAGAUUUCG	GAAGAACACC	AGUGGCGAAG	GCGGCUCACU	GGCUCGAUAC	
	751	761	771	781	791	800
JT-01-b						
	UGACGCUGAG	GUGCGAAAGU	GUGGGGAGCA	AACAGGAUUA	GAUACCCUGG	
	801	811	821	831	841	850
JT-01-b						
	UAGUCCACAC	CGUAAACGAU	GAAUGCCAGU	CGUCGGCAAG	CAUG-CUUGU	
	851	861	871	881	891	900
JT-01-b						
	CGGUGACACA	CCUAACGGAU	UAAGCAUUC	GCCUGGGGAG	UACGGUCGCA	
	901	911	921	931	941	950
JT-01-b						
	AGAUUAAAAC	UCAAAGGAU	UGACGGGGGC	CCGCACAAGC	GGUGGAGCAU	
	951	961	971	981	991	1000
JT-01-b						
	GUGGUUUAAU	UCGAAGCAAC	GCGCAGAACC	UUACCAACCC	UUGACAUCCU	
	1001	1011	1021	1031	1041	1050
JT-01-b						
	CGGACCGCCA	GAGAGAUCUG	GUUUUCACUU	CGGUGACCGA	GUGACAGGUG	

	1051	1061	1071	1081	1091	1100
JT-01-b	CUGCAUGGCU	GUCGUCAGCU	CGUGUCGUGA	GAUGUUCGGU	UAAGUCCGGC	
	1101	1111	1121	1131	1141	1150
JT-01-b	AACGAGCGCA	ACCCACAUCU	UCAGUUGCCA	GCAG-UUC-C	UGGGCACUCU	
	1151	1161	1171	1181	1191	1200
JT-01-b	GGAGAAACUG	CCCGUGAUAA	GCGGGAGGAA	GGUGUGGAUG	ACGUCAAGUC	
	1201	1211	1221	1231	1241	1250
JT-01-b	CUCAUGGCCC	UUACGGGUUG	GGCUACACAC	GUGCUACAAU	GGCAGUGACA	
	1251	1261	1271	1281	1291	1300
JT-01-b	AUGGGU----	-----	-----UAA	UCCCA-AAAA	ACUGUCUCAG	
	1301	1311	1321	1331	1341	1350
JT-01-b	UUCGGAUUGG	GGUCUGCAAC	UCGACCCCAU	GAAGUCGGAA	UCGCUAGUAA	
	1351	1361	1371	1381	1391	1400
JT-01-b	UCGCGUAACA	G-AUGACGCG	GUGAAUACGU	UCCCGGGCCU	UGUACACACC	
	1401	1411	1421	1431	1441	1450
JT-01-b	GCCCGUCACA	CCAGGGGAGU	UGGUUCUACC	CGACGACGCU	GCGCUA....	
	1451	1461	1471	1481	1491	1500
JT-01-b	

	1	11	21	31	41	50
JT-6b
	51	61	71	81	91	100
JT-6bU	AGCUUGC	UA-GAUGACG	
	101	111	121	131	141	150
JT-6b	AGUGGCGGAC	GGGUGAGUAA	UACUUAGGAA	CAUGCCUUUG	UGUGGGGGAU	
	151	161	171	181	191	200
JT-6b	AACUAUUGGA	AACGAUAGCU	AAUACCGCAU	AACGUCUACG	GACCAAAGGG	
	201	211	221	231	241	250
JT-6b	GGC----UUU	G--GCUCUCG	CGCAAAGAGU	GGCCUAAGUG	AGAUUAGCUA	
	251	261	271	281	291	300
JT-6b	GUUGGUGAGG	UAAAGGCUCA	CCAAGGCGAC	GAUCUCUAGC	UGUUCUGAGA	
	301	311	321	331	341	350
JT-6b	GGAAGAUCA	CCACACUGGA	ACUGAGACAC	GGUCCAGACU	CCUACGGGAG	
	351	361	371	381	391	400
JT-6b	GCAGCAGUGG	GGAAUAUUGC	ACAAUGGGGG	AAACCCUGAU	GCAGCCAUGC	
	401	411	421	431	441	450
JT-6b	CGCGUGUGUG	AAGAAGGCCU	UCGGGUUGUA	AAGCACUUUC	AGUUGUGAGG	
	451	461	471	481	491	500
JT-6b	AAAGGUUAGU	AGUUAUACC	UGCUAGCUAU	GACGUUAGCA	ACAGAAGAAG	
	501	511	521	531	541	550
JT-6b	CACCGGCUAA	CUCCGUGCCA	GCAGCCGCGG	UAAUACGGAG	GGUGCGAGCG	
	551	561	571	581	591	600
JT-6b	UUAUUCGGA	UUACUGGGCG	UAAAGCGCAC	GCAGGCGGUU	UGUUAAGCUA	
	601	611	621	631	641	650
JT-6b	GAUGUGAAAG	CCCCGGGCUC	AACCUGGGAA	UAGCAUUUAG	AACUGGCAGA	

	651	661	671	681	691	700
JT-6b	 CUAGAGUCUU	 GGAGAGGGGA	 GUGGAAUUC	 AGGUGUAGCG	 GUGAAAUGCG	
	701	711	721	731	741	750
JT-6b	 UAGAUUUCUG	 GAGGAACAUC	 AGUGGCGAAG	 GCGACUC-CC	 GGCCAAAGAC	
	751	761	771	781	791	800
JT-6b	 UGACGCUCAU	 GUGCGAAAGU	 GUGGGUAGCG	 AACAGGAUUA	 GAUACCCUGG	
	801	811	821	831	841	850
JT-6b	 UAGUCCACAC	 CGUAAACGCU	 GUCUACUAGC	 UGUUUGUGUA	 UUAUUAUCGU	
	851	861	871	881	891	900
JT-6b	 GAGUAGCGAA	 GCUAACGCC-	 UAAGUAGACC	 GCCUGGGGAG	 UACGGCCGCA	
	901	911	921	931	941	950
JT-6b	 AGGUUAAAAC	 UCAAUGAAU	 UGACGGGGGC	 CCGCACAAGC	 GGUGGAGCAU	
	951	961	971	981	991	1000
JT-6b	 GUGGUUUAAU	 UCGAUGCAAC	 GCGAAGAACC	 UUACCUACUC	 UUGACAUCCA	
	1001	1011	1021	1031	1041	1050
JT-6b	 GAAAAUUUGG	 CAGAGAUGCC	 UCAGUGCCUU	 CGGGAAUUCU	 GAGACAGGUG	
	1051	1061	1071	1081	1091	1100
JT-6b	 CUGCAUGGCU	 GUCGUCAGCU	 CGUGUCGUGA	 GAUGUUGGGU	 UAAGUCCCGC	
	1101	1111	1121	1131	1141	1150
JT-6b	 AACGAGCGCA	 ACCCUUGUCC	 UUAGUUGCCA	 GCAU-UUA-U	 UGGGCACUCU	
	1151	1161	1171	1181	1191	1200
JT-6b	 AAGGAGACUG	 CCGGUGACAA	 ACCGGAGGAA	 GGUGGGGACG	 ACGUCAAGUC	
	1201	1211	1221	1231	1241	1250
JT-6b	 AUCAUGGCCC	 UUACGAGUAG	 GGCUACACAC	 GUGCUACAAU	 GGCGAGUACA	
	1251	1261	1271	1281	1291	1300
JT-6b	 GAGGGAAGCG	 AACUUGCGAG	 AGUAAGCGGA	 UCCCUUAAAG	 CUCGUCGUAG	

	1301	1311	1321	1331	1341	1350
JT-6b	UCCGGAUUGG	AGUCUGCAAC	UCGACUCCAU	GAAGUCGGAA	UCGCUAGUAA	
	1351	1361	1371	1381	1391	1400
JT-6b	UCGCAAAUCA	GAAUGUUGCG	GUGAAUACGU	UCCCGGGCCU	UGUACACACC	
	1401	1411	1421	1431	1441	1450
JT-6b	GCCCGUCACA	CCAUGGGAGU	GGGAUGCAAA	ARAA.....	

	1	11	21	31	41	50
JT-08	
	51	61	71	81	91	100
JT-08	...ACAUGCA	AGUCGAGCCG	-----	CCCUUCGG--	-----	GGU
	101	111	121	131	141	150
JT-08	GAGCGGCGGA	CGGGUUAGUA	ACGCGUGGGA	AUAUACCCAA	GGUAGGGAAU	
	151	161	171	181	191	200
JT-08	AGCCUCUGGA	AACGGAGAGU	AAUACCCUAU	GAGCCCUUCG	GGGGAAAGA-	
	201	211	221	231	241	250
JT-08	-----UUU	A-----UCG	CCUUUGGAUU	AGCCCGCGUU	AGAUUAGGUA	
	251	261	271	281	291	300
JT-08	GUUGGUGGGG	UAAUGGCCUA	CCAAGCCUAC	GAUCUAUAGC	UGGUUUUAGA	
	301	311	321	331	341	350
JT-08	GGAUGAUCAG	CAACACUGGG	ACUGAGACAC	GGCCCAGACU	CCUACGGGAG	
	351	361	371	381	391	400
JT-08	GCAGCAGUGG	GGAAUCUUGG	ACAAUGGGCG	CAAGCCUGAU	CCAGCCAUGC	
	401	411	421	431	441	450
JT-08	CGCGUGAGUG	AUGAAGGCCU	UAGGGUCGUA	AAGCUCUUUC	GCCAGGGGAUG	
	451	461	471	481	491	500
JT-08	A-----	---UAA----	-----U	GACAGUACCU	GGUAAAGAAA	
	501	511	521	531	541	550
JT-08	CCCCGGCUAA	CUCCGUGCCA	GCAGCCGCGG	UAAUACGGAG	GGGGUUAGCG	
	551	561	571	581	591	600
JT-08	UUGUUCGGAA	UUACUGGGCG	UAAAGCGCGC	GUAGGCGGAU	CAGAAAGUUG	
	601	611	621	631	641	650
JT-08	GGGGUGAAAU	CCCGGGGCUC	AACCCCGGAA	CUGCCUCCAA	AACUCCUGGU	

	651	661	671	681	691	700
JT-08	CUUGAGUUCG	AGAGAGGUGA	GUGGAAUUC	GAGUGUAGAG	GUGAAAUUCG	
	701	711	721	731	741	750
JT-08	UAGAUUUCG	GAGGAACACC	AGUGGCGAAG	GCGGCUCACU	GGCUCGAUAC	
	751	761	771	781	791	800
JT-08	UGACGCUGAG	GUGCGAAAGU	GUGGGGAGCA	AACAGGAUUA	GAUACCCUGG	
	801	811	821	831	841	850
JT-08	UAGUCCACAC	CGUAAACGAU	GAAUGCCAGU	CGUCAGCAAG	CAUG-CUUGU	
	851	861	871	881	891	900
JT-08	UGGUGACACA	CCUAACGGAU	UAAGCAUUC	GCCUGGGGAG	UACGGUCGCA	
	901	911	921	931	941	950
JT-08	AGAUUAAAAC	UCAAAGGAU	UGACGGGGGC	CCGCACAAGC	GGUGGAGCAU	
	951	961	971	981	991	1000
JT-08	GUUGUUUAAU	UCGAAGCAAC	GCGCAGAACC	UUACCAACCC	UUGACAUC-	
	1001	1011	1021	1031	1041	1050
JT-08	-UAGGACAAC	UCCAGAGAUG	GAGCCUUCU	UCGGGACCUA	GUGACAGGUG	
	1051	1061	1071	1081	1091	1100
JT-08	CUGCAUGGCU	GUCGUCAGCU	CGUGUCGUGA	GAUGUUCGGU	UAAGUCCGGC	
	1101	1111	1121	1131	1141	1150
JT-08	AACGAGCGCA	ACCCACAUC	UUAGUUGCCA	GCAG-UUC-C	UGGGCACUCU	
	1151	1161	1171	1181	1191	1200
JT-08	AGGGAAACUG	CCCGUGAUAA	GCGGGAGGAA	GGUGUGGAUG	ACGUCAAGUC	
	1201	1211	1221	1231	1241	1250
JT-08	CUCAUGGCCC	UUACGGGUUG	GGCUACACAC	GUGCUACAAU	GGCAACUACA	
	1251	1261	1271	1281	1291	1300
JT-08	GUGGGU----	-----	-----UAA	UCCCC-AAAA	GUUGUCUCAG	

	1301	1311	1321	1331	1341	1350
JT-08	UUCGGAUUGG	GGUCUGCAAC	UCGACCCCAU	GAAGUCGGAA	UCGCUAGUAA	
	1351	1361	1371	1381	1391	1400
JT-08	UCGCGUAACA	G-AUGACGCG	GUGAAUACGU	UCCCGGGCCU	UGUACACACC	
	1401	1411	1421	1431	1441	1450
JT-08	GCCCGUCACA	CCAUGGGAGU	UGGGUUUACC	CGAAGGCCGU	GCGCCAACCU	
	1451	1461	1471	1481	1491	1500
JT-08	CG-GGGGGCA	GCGG

	1	11	21	31	41	50
JT-11						

	51	61	71	81	91	100
JT-11						
	..ACATGCAG	TCGAAGCGAT	--GAA--GGG	AGCTTGCTCC	T---GGATTC	
	101	111	121	131	141	150
JT-11						
	AGCGGCGGAC	GGGTGAGTAA	TGCCTAGGAA	TCTGCCTGGT	AGTGGGGGAT	
	151	161	171	181	191	200
JT-11						
	AACGTCCGGA	AACGGGCGCT	AATACCGCAT	ACGTCCTGAG	GGAGAAAGTG	
	201	211	221	231	241	250
JT-11						
	GGGGATCTTC	GGACCTCACG	CTATCAGATG	AGCCTAGGTC	GGATTAGCTA	
	251	261	271	281	291	300
JT-11						
	GTTGGTGGGG	TAAAGGCCTA	CCAAGGCGAC	GATCCGTAAC	TGGTCTGAGA	
	301	311	321	331	341	350
JT-11						
	GGATGATCAG	TCACACTGGA	ACTGAGACAC	GGTCCAGACT	CCTACGGGAG	
	351	361	371	381	391	400
JT-11						
	GCAGCAGTGG	GGAATATTGG	ACAATGGGCG	AAAGCCTGAT	CCAGCCATGC	
	401	411	421	431	441	450
JT-11						
	CGCGTGTGTG	AAGAAGGTCT	TCGGATTGTA	AAGCACTTTA	AGTTGGGAGG	
	451	461	471	481	491	500
JT-11						
	AAGGGCAGTA	AGTTAATACC	TTGCTGTTTT	GACGTTACCA	ACAGAATAAG	
	501	511	521	531	541	550
JT-11						
	CACCGGCTAA	CTTCGTGCCA	GCAGCCGCGG	TAATACGAAG	GGTGCAAGCG	
	551	561	571	581	591	600
JT-11						
	TTAATCGGAA	TTACTGGGCG	TAAAGCGCGC	GTAGGTGGTT	CAGCAAGTTG	
	601	611	621	631	641	650
JT-11						
	GATGTGAAAT	CCCCGGGCTC	AACCTGGGAA	CTGCATCCAA	AACTACTGAG	

	651	661	671	681	691	700
JT-11	CTAGAGTACG	GTAGAGGGTG	GTGGAATTTT	CTGTGTAGCG	GTGAAATGCG	
	701	711	721	731	741	750
JT-11	TAGATATAGG	AAGGAACACC	AGTGGCGAAG	GCGACCACCT	GGACTGATAC	
	751	761	771	781	791	800
JT-11	TGACACTGAG	GTGCGAAAGC	GTGGGGAGCA	AACAGGATTA	GATACCCTGG	
	801	811	821	831	841	850
JT-11	TAGTCCACGC	CGTAAACGAT	GTCGACTAGC	CGTTGGGATC	CTTGAGATCT	
	851	861	871	881	891	900
JT-11	TAGTGGCGCA	GCTAACGCGA	TAAGTCGACC	GCCTGGGGAG	TACGGCCGCA	
	901	911	921	931	941	950
JT-11	AGGTTAAAAC	TCAAATGAAT	TGACGGGGGC	CCGCACAAGC	GGTGGAGCAT	
	951	961	971	981	991	1000
JT-11	GTGGTTTAAT	TCGAAGCAAC	GCGAAGAACC	TTACCTGGCC	TTGACATGCT	
	1001	1011	1021	1031	1041	1050
JT-11	GAGAACTTTC	CAGAGATGGA	TTGGTGCCTT	CGGGAActCA	GACACAGGTG	
	1051	1061	1071	1081	1091	1100
JT-11	CTGCATGGCT	GTCGTCAGCT	CGTGTCTGTA	GATGTTGGGT	TAAGTCCCGT	
	1101	1111	1121	1131	1141	1150
JT-11	AACGAGCGCA	ACCCTTGTCC	TTAGTTACCA	GCACCTCGGG	TGGGCACTCT	
	1151	1161	1171	1181	1191	1200
JT-11	AAGGAGACTG	CCGGTGACAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	
	1201	1211	1221	1231	1241	1250
JT-11	ATCATGGCCC	TTACGGCCAG	GGCTACACAC	GTGCTACAAT	GGTCGGTACA	
	1251	1261	1271	1281	1291	1300
JT-11	AAGGGTTGCC	AAGCCGCGAG	GTGGAGCTAA	TCCCATAAAA	CCGATCGTAG	

	1301	1311	1321	1331	1341	1350
JT-11	TCCGGATCGC	AGTCTGCAAC	TCGACTGCGT	GAAGTCGGAA	TCGCTAGTAA	
	1351	1361	1371	1381	1391	1400
JT-11	TCGTGAATCA	GAATGTCACG	GTGAATACGT	TCCCGGGCCT	TGTACACACC	
	1401	1411	1421	1431	1441	1450
JT-11	GCCCCGTCACA	CCATGGGAGT	GGGTTGCTCC	AGAAGTAGCT	AGTCTAACCG	
	1451	1461	1471	1481	1491	1500
JT-11	CAAGGGGG..
	1501	1511	1521	1531	1541	
JT-11	

	1	11	21	31	41	50
JT-21						

	51	61	71	81	91	100
JT-21						
CAGT	C--GAGTATT	
	101	111	121	131	141	150
JT-21						
	GATAATGCCC	GGGTGCGTAA	CGCGTATGCA	CCTACCTGTT	ACAGGGGAAT	
	151	161	171	181	191	200
JT-21						
	AGCCCGGGGA	AACCCGGATT	AATATCCCAT	AGGACTGCAT	GGTTAAAGC-	
	201	211	221	231	241	250
JT-21						
	-----CTT	G-----GTG	GTAACAGACG	GGCATGCGTA	GGATTAGCTA	
	251	261	271	281	291	300
JT-21						
	GTTGGAGAGG	TAACGGCTCA	CCAAGGCGAC	GATCCTTAGG	GGTTCTGAGA	
	301	311	321	331	341	350
JT-21						
	GGAAGGTCCC	CCACACTGGC	ACTGAGATAC	GGGCCAGACT	CCTACGGGAG	
	351	361	371	381	391	400
JT-21						
	GCAGCAGTAG	GGAATATTGG	TCAATGGGCG	AGAGCCTGAA	CCAGCCATGC	
	401	411	421	431	441	450
JT-21						
	CGCGTGCAGG	AAGACGGCGT	CTGCGTTGTA	AACTGCTTTT	ATCTGGGAAG	
	451	461	471	481	491	500
JT-21						
	AA-AAGCCCT	---TGCG---	-AGGGAAATT	GCCGGTACCA	GAAGAATAAG	
	501	511	521	531	541	550
JT-21						
	CACCGGCTAA	CTCCGTGCCA	GCAGCCGCGG	TAATACGGAG	GGTGCAAGCG	
	551	561	571	581	591	600
JT-21						
	TTGTCCGGAT	TTATTGGGTT	TAAAGGGTGC	GTAGGCGGCT	GATTAAGTCA	
	601	611	621	631	641	650
JT-21						
	GCGGTGAAAG	TTCTCGGCTC	AACCGAGAGA	CTGCCGTTGA	TACTGGTCAG	

	651	661	671	681	691	700
JT-21	CTTGAGTTAT	GGAGGGGTAC	ATGGAATTTA	TGGTGTAGCG	GTGAAATGCA	
	701	711	721	731	741	750
JT-21	TAGATACCAT	AAGGAACACC	GATAGCGAAG	GCATTGTACT	GGCCATAAAC	
	751	761	771	781	791	800
JT-21	TGACGCTGAG	GCACGAAAGC	GTGGGTAGCG	AACAGGATTA	GATACCCTGG	
	801	811	821	831	841	850
JT-21	TAGTCCACGC	CGTAAACGAT	GATCACTCGC	TTTCATCG--	-TAGT--AAT	
	851	861	871	881	891	900
JT-21	TTGAGGCCAA	GCGAAAGCGT	TAAGTGATCC	ACCTGGGGAG	TACGCCGGCA	
	901	911	921	931	941	950
JT-21	ACGGTGAAAC	TCAAAGGAAT	TGACGGGGGT	CCGCACAAGC	GGTGAGCAT	
	951	961	971	981	991	1000
JT-21	GTGGTTTAAT	TCGATGATAC	GCGAGGAACC	TTACCCGGGC	TAGAATGTAA	
	1001	1011	1021	1031	1041	1050
JT-21	GGAATGATAC	AAGATGTGTC	AGTC--AGCA	ATGA--CCTG	AAACAAGGTG	
	1051	1061	1071	1081	1091	1100
JT-21	CTGCATGGTT	GTCGTCAGCT	CGTGCCGTGG	AGTGTTGGTT	TAAGTCCCGC	
	1101	1111	1121	1131	1141	1150
JT-21	AACGAGCGCA	ACCCCTGTTG	TCAGTTGCCA	TCAAGTAATT	TGGGGACTCT	
	1151	1161	1171	1181	1191	1200
JT-21	GACAAGACTG	CCCGCG-CAA	GC-GGAG-A.	

	451	461	471	481	491	500
JT-22-a
	501	511	521	531	541	550
JT-22-a	GGGUUAGCCG	
	551	561	571	581	591	600
JT-22-a	UUGUUCGGAA	UUACUGGGCG	UAAAGCGCGC	GUAGGCGGAU	CAGAAAGUUG	
	601	611	621	631	641	650
JT-22-a	GGGGUGAAAU	CCCAGGGCUC	AACCCUGGAA	CUGCCUCCAA	AACUCCUGGU	
	651	661	671	681	691	700
JT-22-a	CUUGAGUUCG	AGAGAGGUGA	GUGGAACUCC	GAGUGUAGAG	GUGAAAUUCG	
	701	711	721	731	741	750
JT-22-a	UAGAUUUCG	GAAGAACACC	AGUGGCGAAG	GCGGCUCACU	GGCUCGAUAC	
	751	761	771	781	791	800
JT-22-a	UGACGCUGAG	GUGCGAAAGU	GUGGGGAGCA	AACAGGAUUA	GAUACCCUGG	
	801	811	821	831	841	850
JT-22-a	UAGUCCACAC	CGUAAACGAU	GAAUGCCAGU	CGUCGGGUAG	UAUA-CUAUU	
	851	861	871	881	891	900
JT-22-a	CGGUGACACA	CCUAACGGAU	UAAGCAUUC	GCCUGGGGAG	UACGGUCGCA	
	901	911	921	931	941	950
JT-22-a	AGAUUAAAAC	UCAAAGGAAU	UGACGGGGGC	CCGCACAAGC	GGUGGAGCAU	
	951	961	971	981	991	1000
JT-22-a	GUGGUUUAAU	UCGAAGCAAC	GCGCAGAACC	UUACCAACCC	UUGACAUCU	
	1001	1011	1021	1031	1041	1050
JT-22-a	GAUCGCGGGA	UCGUAGAGAU	ACUUUCCUUC	AGUGGGAUCA	GUGACAGGUG	
	1051	1061	1071	1081	1091	1100
JT-22-a	CUGCAUGGCU	GUCGUCAGCU	CGUGUCGUGA	GAUGUUCGGU	UAAGUCCGGC	

	1101	1111	1121	1131	1141	1150
JT-22-a	AACGAGCGCA	ACCCACAUCC	CUAGUUGCCA	GCAG-UUC-C	UGGGCACUCU	
	1151	1161	1171	1181	1191	1200
JT-22-a	AUGGAAACNG	CCCGUGAUAA	GCGGGAGGGA	AGUUGGAUUG	ACUUCAAGUC	
	1201	1211	1221	1231	1241	1250
JT-22-a	CUCAUGG-CC	CUACGGGUUG	GGCUCCACAC	GUGCUACAUU	GGCAGUGACA	
	1251	1261	1271	1281	1291	1300
JT-22-a	AAGGGU----	-----	-----UAA	UCCCA-AAAA	ACUG.....	

	1	11	21	31	41	50
JT-23
	51	61	71	81	91	100
JT-23	.CACATGCAA	GTCGAACGGT	GAACA-TGAA	AGACTTTCTT	TT-GATGACG	
	101	111	121	131	141	150
JT-23	AGTGGCGGAC	GGGTGAGTAA	CACGTGGGAA	TCTACCGAGT	AGCGGGGGAT	
	151	161	171	181	191	200
JT-23	AGCCCGGAGA	AATCCGGATT	AATACCGCAT	AAGCACTACG	GTGTAAAGGG	
	201	211	221	231	241	250
JT-23	GGCCTTCTTG	AAAGCTTCCA	CTATTGATG	AGCCTGCGTC	AGATTAGCTA	
	251	261	271	281	291	300
JT-23	GTTGGTGGGG	TAATGGCCTA	CCAAGGCGAC	GATCTGTAGC	TGGTCTGAGA	
	301	311	321	331	341	350
JT-23	GGATGATCAG	CCACACTGGG	ACTGAGACAC	GGCCCAGACT	CCTACGGGAG	
	351	361	371	381	391	400
JT-23	GCAGCAGTGG	GGAATATTGG	ACAATGGGGG	CAACCCTGAT	CCAGCAATAC	
	401	411	421	431	441	450
JT-23	CGCGTGTGTG	AAGAAGGCCT	TCGGGTTGTA	AAGCACTTTC	AGCGAGGAGG	
	451	461	471	481	491	500
JT-23	AAAAGCTAGT	AGTTAATACC	TCTGGCCTTT	GACGTTACTC	GCAGAAGAAG	
	501	511	521	531	541	550
JT-23	CACCGGCTAA	CTCCGTGCCA	GCAGCCGCGG	TAATACGGAG	GGTGCCAGCG	
	551	561	571	581	591	600
JT-23	TTAATCGGAA	TTACTGGGCG	TAAAGCGCGC	GTAGGCGGAT	GATTAAGTCA	
	601	611	621	631	641	650
JT-23	GATGTGAAAG	CCCAGGGCTT	AACCTTGGA	CTGCATTTGA	TACTGGTCAT	

	651	661	671	681	691	700
JT-23	CTAGAGTACA	GAAGAGGGGG	GTGGAATTCC	AGGTGTAGCG	GTGAAATGCG	
	701	711	721	731	741	750
JT-23	TAGATATCTG	GAGGAACATC	AGTGGCGAAG	GCGACCCCTT	GGTCTGATAC	
	751	761	771	781	791	800
JT-23	TGACGCTGAG	GTGCGAAAGC	GTGGGTAGCG	AACAGGATTA	GATACCCTGG	
	801	811	821	831	841	850
JT-23	TAGTCCACGC	CGTAAACGAT	GTCTATTAGC	TGTTGGGTAT	-ATTAAGAAC	
	851	861	871	881	891	900
JT-23	TAGTGGCGC-	GCTAACGCTT	TAAATAGACC	GCCTGGGGAG	TACGGCCGCA	
	901	911	921	931	941	950
JT-23	AGGTTAAAAC	TCAAATGAAT	TGACGGGGGC	CCGCACAAGC	GGTGGAGCAT	
	951	961	971	981	991	1000
JT-23	GTGGTTTAAT	TCGATGCAAC	GCGAAGAACC	TTACCAGGTC	TTGACATCCA	
	1001	1011	1021	1031	1041	1050
JT-23	GAGAACTTTC	CAGAGATGGA	TTGGTGCCTT	CGGGAACCTC	GTGACAGGTG	
	1051	1061	1071	1081	1091	1100
JT-23	CTGCATGGCT	GTCGTCAGCT	CGTGTCGTGA	AGTGTTGGGT	TAAGTCCCGT	
	1101	1111	1121	1131	1141	1150
JT-23	AACGAGCGCA	ACCCTTGTCC	TTAGTTGCTA	ACAT-TTAAT	TGAGAACTCT	
	1151	1161	1171	1181	1191	1200
JT-23	AAGGAGACTG	CCGGTGACAA	ACCGGAGGGA	AGGGGGGACG	ACGTCAAGTC	
	1201	1211	1221	1231	1241	1250
JT-23	ATCATGGCCC	TTACGACCTG	GGCTCCACAC	GTGCTACATG	GGGCAGT-CA	
	1251	1261	1271	1281	1291	1300
JT-23	GA----A...

	551	561	571	581	591	600
JT-27-cCGCGC	GTAGGCGGCT	TGATAAGCCG	
	601	611	621	631	641	650
JT-27-c	GTTGTGAAAG	CCCTGGGCTC	AACCTGGGAA	CGGCATCCGG	AACTGTCAGG	
	651	661	671	681	691	700
JT-27-c	CTAGAGTGCA	GGAGAGGAAG	GTAGAATTCC	CGGTGTAGCG	GTGAAATGCG	
	701	711	721	731	741	750
JT-27-c	TAGAGATCGG	GAGGAATACC	AGTGGCGAAG	GCGGCCTTCT	GGACTGACAC	
	751	761	771	781	791	800
JT-27-c	TGACGCTGAG	GTGCGAAAGC	GTGGGTAGCA	AACAGGATTA	GATACCCTGG	
	801	811	821	831	841	850
JT-27-c	TAGTCCACGC	CGTAAACGAT	GTCGACTAGC	CGTTGGGCTC	CTTGAGAGCT	
	851	861	871	881	891	900
JT-27-c	TTGTGGCGCA	GTAAACGCGA	TAAGTCGACC	GCCTGGGGAG	TACGGCCGCA	
	901	911	921	931	941	950
JT-27-c	AGGTTAAAAC	TCAAATGAAT	TGACGGGGGC	CCGCACAAGC	GGTGGAGCAT	
	951	961	971	981	991	1000
JT-27-c	GTGGTTTAAT	TCGATGCAAC	GCGAAGAACC	TTACCTACCC	TTGACATCGA	
	1001	1011	1021	1031	1041	1050
JT-27-c	GAGAACTTTC	CAGAGATGGA	TTGGTGCCTT	CGGGANCTCT	CAGACAGGTG	
	1051	1061	1071	1081	1091	1100
JT-27-c	CTGCATGGCT	GTCGTCAGCT	CGTGTGTGA	AATGTTGGGT	TAAGTCCCGT	
	1101	1111	1121	1131	1141	1150
JT-27-c	AACGAGCGCA	ACCCTTGTC	CTATTTGCCA	GCGA-TTC-T	CGGGAACCTCT	
	1151	1161	1171	1181	1191	1200
JT-27-c	AGGGGAGACTG	CCGGTGACAA	ACCGGAGGAA	GGTGGGGACG	ACGTCAAGTC	

	1201	1211	1221	1231	1241	1250
JT-27-c	ATCATGGCCC	TTACGGGTAG	GGCTACACAC	GTGCTACAAT	GGCCGGTACA	
	1251	1261	1271	1281	1291	1300
JT-27-c	ATGGGTTGCG	AATCCGCGAG	GTGGAGCTAA	TCCCATAAAG	CCGGTCTCAG	
	1301	1311	1321	1331	1341	1350
JT-27-c	TCCGGATCGG	AGTCTGCAAC	TCGACTCCGT	GAAGTCGGAA	TCGCTAGTAA	
	1351	1361	1371	1381	1391	1400
JT-27-c	TCGTGATTCA

	1	11	21	31	41	50
JT-29
	51	61	71	81	91	100
JT-29UGCAA	GUCGAGCCGA	AACGA-UUCU	AGCUUGCUAG	AA-GGCGUCG	
	101	111	121	131	141	150
JT-29	AGCGGCGGAC	GGGUGAGUAA	UGCAUGGGAA	UCUGCCCGAU	AGUGGGGGAC	
	151	161	171	181	191	200
JT-29	AACCUGGGGA	AACUCAGGCU	AAUACCGCAU	ACGUCCUACG	GGAGAAAGCA	
	201	211	221	231	241	250
JT-29	GGGGAUCUUC	GGACCUUGCG	CUAUCGGAUG	AGCCCAUGUC	GGAUUAGCUU	
	251	261	271	281	291	300
JT-29	GUUGGUGAGG	UAACGGCUCA	CCAAGGCGAC	GAUCCGUAGC	UGGUCUGAGA	
	301	311	321	331	341	350
JT-29	GGAUGAUCAG	CCACACUGGG	ACUGAGACAC	GGCCCAGACU	CCUACGGGAG	
	351	361	371	381	391	400
JT-29	GCAGCAGUGG	GGAAUAUUGG	ACAAUGGGCG	AAAGCCUGAU	CCAGCCAUGC	
	401	411	421	431	441	450
JT-29	CGCGUGUGUG	AAGAAGGCCU	UCGGGUUGUA	AAGCACUUUC	AGCGAGGAAG	
	451	461	471	481	491	500
JT-29	AACGCUUCGG	GAUUAUACU	CCCAGGAAA	GACAUACUC	GCAGAAGAAG	
	501	511	521	531	541	550
JT-29	CACCGGCUAA	CUCCGUGCCA	GCAGCCGCGG	UAAUACGGAG	GGUGCAAGCG	
	551	561	571	581	591	600
JT-29	UUAAUCGGAA	UUACUGGGCG	UAAAGCGCGC	GUAGGUGGCU	AAGUCAGCCA	
	601	611	621	631	641	650
JT-29	GGUGUGAAAG	CCCCGGGCUC	AACCUGGGAA	CGGCAUCUGG	AACUGCUUGG	

	651	661	671	681	691	700
JT-29	CUAGAGUGCA	GGAGAGGAAG	GUAGAAUUC	CGGUGUAGCG	GUGAAAUGCG	
	701	711	721	731	741	750
JT-29	UAGAGAUCCG	GAGGAAUACC	AGUGGCGAAG	GCGGCCUUCU	GGACUGACAC	
	751	761	771	781	791	800
JT-29	UGACACUGAG	GUGCGAAAGC	GUGGGUAGCA	AACAGGAUUA	GAUACCCUGG	
	801	811	821	831	841	850
JT-29	UAGUCCACGC	CGUAAACGAU	GUCAACUAGC	CGUUGGGUCC	CUUGAGGACU	
	851	861	871	881	891	900
JT-29	UAGUGGCGCA	GCUAACGCAA	UAAGUUGACC	GCCUGGGGAG	UACGGCCGCA	
	901	911	921	931	941	950
JT-29	AGGUUAAAAC	UCAAUGAAU	UGACGGGGGC	CCGCACAAGC	GGUGGAGCAU	
	951	961	971	981	991	1000
JT-29	GUGGUUUAAU	UCGAUGCAAC	GCGAAGAACC	UUACCUACCC	UUGACAUCCA	
	1001	1011	1021	1031	1041	1050
JT-29	GAGGACUUUC	CAGAGAUGGA	UUGGUGCCUU	CGGGAACUCU	GAGACAGGUG	
	1051	1061	1071	1081	1091	1100
JT-29	CUGCAUGGCU	GUCGUCAGCU	CGUGUUGUGA	AAUGUUGGGU	UAAGUCCCGU	
	1101	1111	1121	1131	1141	1150
JT-29	AACGAGCGCA	ACCCCUAUCC	UUAUUUGCCA	GCGAGUAAUU	CGGGAACUCU	
	1151	1161	1171	1181	1191	1200
JT-29	AAGGAGACUG	CCGGA-----	-----	-----	-----	
	1201	1211	1221	1231	1241	1250
JT-29	-UCAUGGCCC	UUACGGGUAG	GGCUACACAC	GUGCUACAAU	GGCAAGUACA	
	1251	1261	1271	1281	1291	1300
JT-29	AAGGGUUGCA	AUACGGCGAC	GUGGAGCCAA	UCCCAUAAAG	CUUGCCUCAG	

	1301	1311	1321	1331	1341	1350
JT-29	UCCGGAUUGG	AGUCUGCAAC	UCGACUCCAU	GAAGUCGGAA	UCGCUAGUAA	
	1351	1361	1371	1381	1391	1400
JT-29	UCGUGGAUCA	GAAUGCCACG	GUGAAUACGU	UCCCGGGCCU	UGUACACACC	
	1401	1411	1421	1431	1441	1450
JT-29	GCCCGUCACA	CCAUGGGAGU	GGACUGCACC	AGAAGUGGUU	AGCCUAACCU	
	1451	1461	1471	1481	1491	1500
JT-29	UCGGGAG...	*****	*****	*****	*****	*****